Journal of DAIRY SCIENCE

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JOURNAL OF DAIRY SCIENCE

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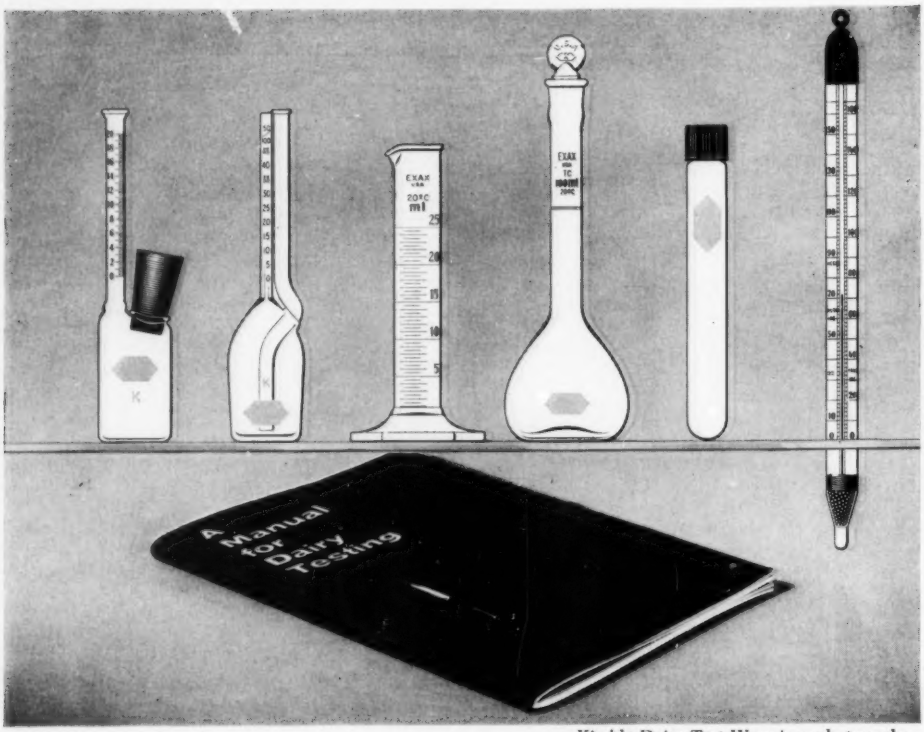

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PEOPLE AND EVENTS

53rd Annual A.D.S.A. Meeting Hosts—North Carolina State College

The university staff and people of the dairy industry in North Carolina are making elaborate plans for the 53rd Annual Meeting of the American Dairy Science Association. Excellent facilities are being made available for holding the sectional meetings and for providing entertainment traditional to the state of North Carolina. Convention visitors will also have an opportunity to see places of historic interest in the area. Pictured above are members of the steering committee responsible for making local plans and arrangements.

Left to right are (front row) J. W. POU, G. H. WISE, C. WHITESIDES, manager of the North Carolina Milk Producers Association, W. R. MURLEY, and W. M. ROBERTS; (back row) M. SENGER, E. G. BATTE, M. L. SPECK, L. LANGDON, executive vice-president of the North Carolina Dairy Products Association, F. G. WARREN, E. R. BARRICK, M. B. WISE, and T. C. BLALOCK.

Welcome to Members of the American Dairy Science Association

We are looking forward to having the 53rd annual meeting of the American Dairy Science Association on our campus in June, 1958.

Having this convention at North Carolina State College will bring many benefits to our staff and the dairy industry of the state. We consider it a privilege and an opportunity to be your hosts and shall strive to make your stay pleasant and profitable.

We are proud of the growth and progress of the dairy industry in the state, which has resulted from the joint efforts of every segment of dairying and the college. Your coming to our state will provide encouragement and inspiration for moving further ahead in our programs of teaching, research, and extension.

North Carolina offers unusual opportunities for pleasant vacations. We hope you will combine your coming to Raleigh for the meetings with a sojourn at one of our many attractive beaches or in the mountains.

Every member of our staff wants your stay to be enjoyable and worthwhile. Please let us know if we can do anything to assist you in making your visit more pleasant.

We shall take delight in being your hosts and extend a cordial welcome to you.

CAREY H. BOSTIAN, *Chancellor*
North Carolina State College

Michigan News

Three Michigan State Univ. dairy faculty members participated in the annual meeting of the Upper Peninsula Dairy Manufacturers, Jan. 21-23, at Marquette. They were D. L. MURRAY, C. J. LITTLE, and G. M. TROUT. F. M. SKIVER, chief of the Bureau of Dairying, along with other dairy people in Michigan also participated.

C. M. STINE has been appointed assistant professor of dairy manufactures. Dr. Stine received his B.S. and M.S. degrees from Pennsylvania State Univ., University Park, and his Ph.D. degree from the Univ. of Minnesota, St. Paul. He will teach courses in cheesemaking and edible fats and oils at Michigan State.

The Saginaw–Bay City Dairy Technology Society met in January with C. W. HALL, professor of agricultural engineering at M.S.U.,

as principal speaker. His subject was "Making Your Plants Efficient."

L. G. HARMON, associate professor of dairying at M.S.U., has been elected secretary of the Detroit Dairy Technology Society. He succeeds P. S. LUCAS of the Dairy Dept. at M.S.U., who guided the Detroit Society through its founding period and has served as its secretary for the past 12 years.

U.S.—Thailand Milk Program Feted

Movie cameras whirled and flash bulbs flared at special ceremonies in Far East Thailand, when U. S. and Thailand government officials gathered at La-or-Utit School in Bangkok for ceremonies marking the one-millionth bottle of milk given to Thailand school children. Into the eager hands of dainty CHARUWAN THARTRAKORN, a kindergartner, went the coveted gold-painted bottle. At the special event commemorating the millionth bottle of milk, POTE SARASIN, Prime Minister of Thailand, presented the milk, which comes through the U. S. Foreign Agricultural Service–Dairy Society International (DSI) program in Thailand.

The Thailand Dairy Program, under the direction of Mrs. DOROTHY MCCANN of Chicago, a DSI representative, was first inaugurated at the great Bangkok Constitutional Fair a year ago, and the recombined milk—made in Bangkok from U. S. dry ingredients—is furnished under contract by Foremost Dairy, Ltd., of Bangkok—a Thai-U. S. financed corporation. The program is a part of the continuing program to introduce the use of milk and its products in milk-deficient areas, through the use of recombined milk and other dairy products. The distribution is backed up by a campaign for broader knowledge of all dairy products.

University of Georgia News

About 125 people attended the annual fall banquet of the Dairy Science Club, which was held on Dec. 6. The speaker was PERRY ELLSWORTH of the Milk Industry Foundation. The new recruitment film for the dairy industry, "This Is the Dairy Industry," was shown.

H. B. HENDERSON, Head of the Dairy Department, presented the Borden Foundation scholarship to J. CASEY, a tuition scholarship to RONALD WADDELL, and $200 scholarships, donated by individual dairy organizations in the state, to the following students: R. ADAMSON, R. BISHOP, B. BOWELL, L. D. COWART, R. HALEY, G. HERRING, M. HOEFLIN, D. JOHNSON, W. JOHNSON, J. LEE, J. MCLEOD, F. MILLER, E. MOORE, J. RHENEY, and F. STROUPE. In addition, $200 scholarships were donated by Sears Roebuck & Company to J. CARLTON and H. D. WILLIAMS.

J. J. SHEURING was elected second vice-president of the International Association of Milk and Food Sanitarians at its last annual meeting. By virtue of holding this office, he is slated to succeed to the presidency in 1960.

At the last session of the Georgia legislature, a bill for the registration of professional sanitarians was passed. The board of examiners consists of J. J. SHEURING, *Chairman*, D. BELCHER, G. H. DEHART, J. W. MANN, and L. C. WILLIAMS. The committee has been very active in processing applications.

The one-day dairy production short course was held January 28 at Carrollton, January 29 at Rock Eagle 4-H Center, Eatonton, and January 30 at Tifton. K. M. AUTREY of Alabama Polytechnic Institute, GEORGE H. HOPSON of DeLaval Separator Co., S. L. KALISON of Virginia Polytechnic Institute, and H. K. WELCH, JOHN S. CONNER, and HOWARD L. DALTON of the Univ. of Georgia were on the program.

H. B. HENDERSEN was one of the four judges in the ice cream clinic held at the convention of the Southern Association of Ice Cream Manufacturers at Hollywood, Fla., in December.

Oregon News

The Oregon Dairyman's Association held their 64th annual meeting in Klamath Falls, Dec. 2-4.

The new brucellosis law, the milk audit law, and bulk handling of milk were highlighted during the session. L. GERAGHTY, Klamath Falls, was elected president; L. WETTSTEIN, Ontario, was named vice-president; H. P. EWALT, extension dairyman, secretary, and F. ROBERTS, Oregon Dairy Breeders, treasurer.

W. LYON, Junction City, was elected president of the Oregon State Guernsey Breeders Association at their annual meeting Dec. 6 at Corvallis.

S. SPENCER, O.S.C. student and Guernsey breeder, was elected vice-president; B. A. NEWELL, Marion County extension agent, was named secretary, and H. B. HOWELL, superintendent of the John Jacob Astor Experiment Station, was elected treasurer.

The Oregon Milk and Food Sanitarians held their annual meeting at Corvallis, Dec. 19. E. RACKLEFF and G. POE presented reports on the National meeting and H. W. SCHULTZ, head of the Dept. of Food and Dairy Technology at Oregon State College, Corvallis, spoke on "The Challenge to the Dairy Technologist."

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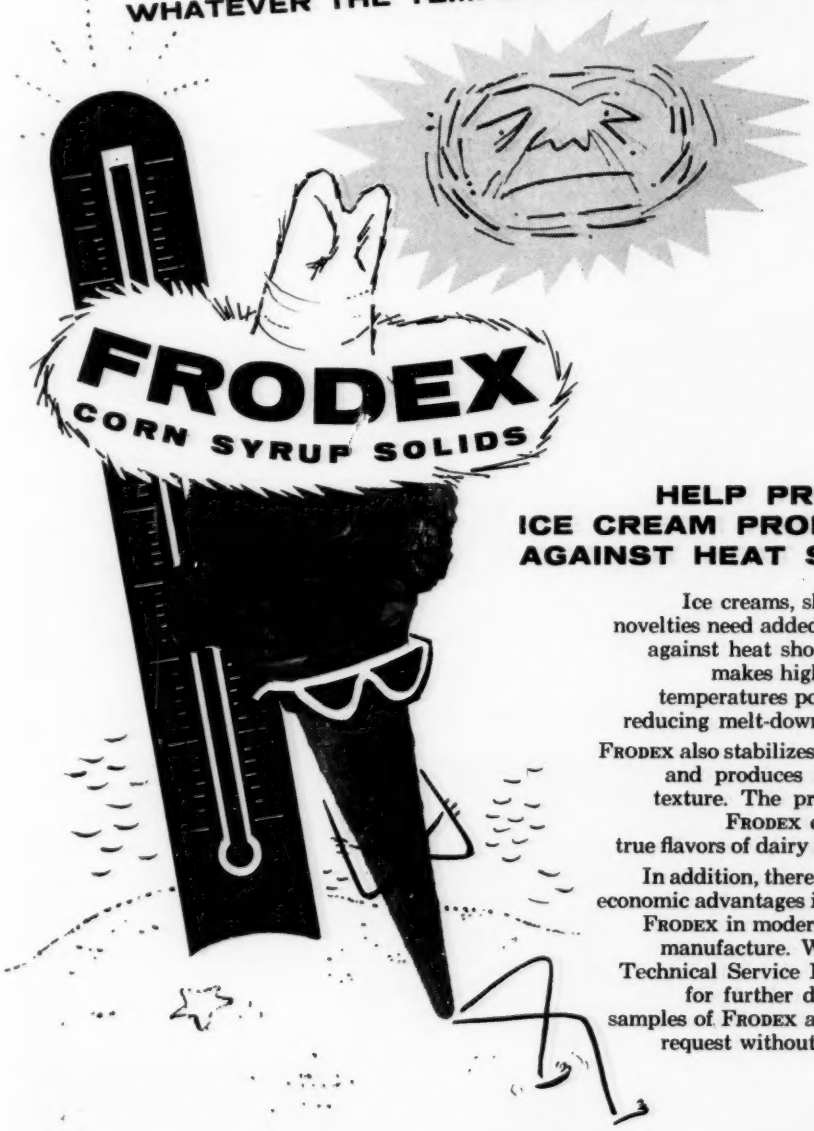

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The second annual Oregon Dairy Council program was attended by some 80 processors and producers in Portland, Dec. 6. G. Loy, Oregon Dairy Council manager, arranged the program.

Directors elected for the council were O. Feldkamp, Roseburg; H. Gallagher, Portland; L. Albright, McMinnville; F. Rood, North Bend; K. Green and B. Doods, Portland; E. Peterson, Scappoose; E. Hanke, Astoria, and R. Garrison, Eugene. Rood was elected president for 1958.

New Editor for Student Chapter News Section

E. L. Thomas, of the Univ. of Minnesota, St. Paul, resigned as Editor of the Student Chapter News Section, effective Dec. 31, 1957. Chiefly through the efforts of Dr. Thomas, this section has earned a permanent place in the Journal. He has created a real interest in the student affiliate program by submitting timely news from student chapters.

Beginning January 1, 1958, J. E. Johnston, of Louisiana State Univ., began editing the student section of the Journal. He is intensely interested in this program for students and will do everything possible to stimulate their interests in Dairy Science.

The A.D.S.A. appreciates the leadership of Dr. Thomas during the past two years and extends thanks for a job well done. To Dr. Johnston, the Association extends support and encouragement to inspire a permanent interest in dairy science among students, both undergraduates and graduates.

Help Us to Help You

Yes, your Association needs your *help* if it is to do the best job of *helping* you. Have you ever fully realized the *great* job that the A.D.S.A. is doing for you, the dairy industry, and our country? Our contributions are very meager when one considers the tremendous task before us and the accomplishments of our association. In addition to the monthly publication of the Journal, and the annual meetings, your association represents you and our industry on numerous national committees and is constantly involved in policy-making matters that affect the entire dairy industry.

Your Association needs the active support of all the leaders in the dairy industry if it is to keep pace with the rapid changes that are taking place in America. Never before in the history of our association has it been so important that we have a strong, active organization.

There is a way for you to serve your Association. The A.D.S.A. needs good representation in all segments of the industry. With increased representation, your association can

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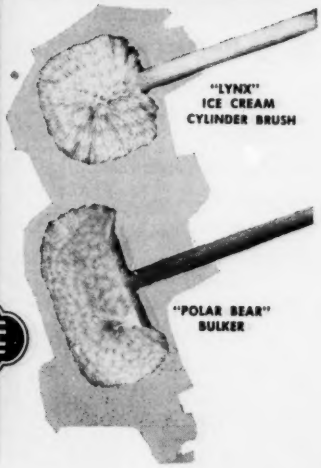

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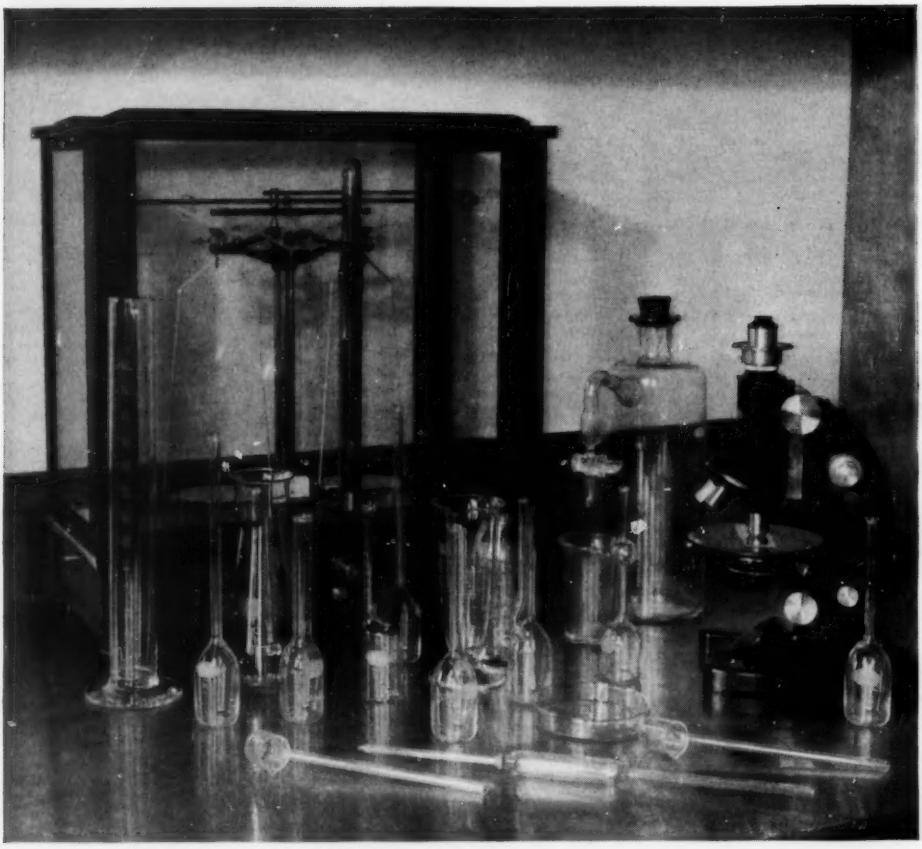

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speak more authoritatively for the dairy industry and also provide increased services to its members. The officers of the A.D.S.A. and your membership committee would like for you to help in *membership promotion* by making personal contacts with people that you know are eligible for membership. Just think how strong our association would be if everyone would adopt this "Self-Help" attitude.

J. B. FRYE, JR., *Chairman*
National Membership Committee

Montana News

More than 100 dairy industry people from the Pacific Northwest attended the Twenty-second Annual Dairy Industry Short Course and Dairy Products Show, held Nov. 18-23 at Montana State College, Bozeman.

Guest speakers were N. C. ANGEVINE, Meyer-Blanke Co., St. Louis, Mo.; H. BOWSER, Dairypak, Cleveland, O.; H. E. CALBERT, Univ. of Wisconsin, Madison; M. CLARK, American Dairy Association, Chicago, Ill.; N. E. LAZARUS, Lazarus Laboratories, Buffalo, N. Y.; E. PIRNER, Marathon Corp., Menasha, Wis.; I. W. SLATER, Univ. of Wyoming, Laramie, and L. H. MINOR, Wyandotte Chemicals Corp.

Clinics were held on butter, cheese, ice cream, and milk; and a clinic on the commercial manufacture of cottage cheese was held, with Mr. Angevine as instructor. Seventy-eight samples of dairy products were entered in the Dairy Products Show and 24 prizes and certificates of merit were awarded

---

E. A. KEYES, professor of dairy industry at Montana State for the past 12 years, has joined the Univ. of Illinois team to teach in the Rafi Abmad Kidwai Agricultural Institute, Sehore, M.P., India. Dr. Keyes left in December, 1957, for a two-year assignment.

News from Ohio

The Seventh Annual Ohio Milk Sanitation and Public Health Short Course will be held March 17-22, 1958, under the cooperative sponsorship of the Ohio Dept. of Health, the Ohio Dept. of Agriculture, and the Ohio State Univ., Columbus.

Cleaning and sanitizing in the plant and on the farm, HTST operation, plant and farm inspections, farm water supplies, and product quality control will be the major areas of discussion.

The program on March 21 will be devoted to problems in plant sanitation and quality control of particular interest to the dairy plant operator. This is the first year such a program has been included in this particular short course.

J. J. BETSCHER has been appointed instructor in the Dairy Technology Dept. Mr. BETSCHER received his B.S. and M.S. degrees from Ohio State and is currently conducting research on butter spreadability.

REX PAXTON, director of public relations for the Sutherland Paper Company, was the principal speaker at the January meetings of the Maumee Valley, Northeastern Ohio, Central Ohio, and Cincinnati Dairy Technology Societies. His topics was "Meeting the Challenge."

The Silver Jubilee Meeting of the Ohio Dairy Technology Conference is scheduled for Feb. 25-28 on the Ohio State Univ. campus at Columbus. Approximately 35 speakers from educational, regulatory, and industrial institutions will participate in the event. The three-day session will include a technical program and a

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Silver Jubilee banquet, the banquet to be held Feb. 27.

Four major areas of interest to be covered in the technical program are manufacturing, field work, market-milk, and ice cream. The manufacturing section will be concerned with pesticides, sanitation, water supplies, and new dairy products. The field work section will discuss problems of quality, economics, and producer-plant relations. The market-milk section will discuss quality, plant efficiency, and "men and machines of the future." The ice cream section will feature discussions on quality, homogenization efficiency, nut ice cream, and new flavors for ice cream and sherbet.

The Jubilee banquet will be sponsored cooperatively by the Dept. of Dairy Technology and the four Dairy Technology Societies of Ohio (Maumee Valley, Northeastern Ohio, Central Ohio, and Cincinnati).

G. J. KIENZLE, vice-president in charge of public relations of Borden's Midwest Division, has been named director of the O.S.U. School of Journalism. His appointment became effective Jan. 1, 1958.

An alumnus of O.S.U., Mr. Kienzle has had ten years of experience as a newspaperman with the Columbus *Dispatch* and the Associated Press, in addition to 13 years of experience in public relations with Firestone Tire and Rubber Co. and with Borden's.

A. D. PRATT, associate professor of dairy science, Ohio Agricultural Experiment Station, Wooster, Ohio, has left the United States to undertake a one-year assignment as animal nutritionist in Argentina, under the Expanded Technical Assistance Program of the Food and Agriculture Organization of the United Nations.

Tennessee News

Two of the three $300 scholarships annually awarded at the Univ. of Tennessee to juniors majoring in dairy manufacturing have been awarded to J. SCOTT and H. HOLT. The third scholarship will be awarded this spring. These scholarships were established in 1957 by the Tennessee Dairy Products Association for the purpose of encouraging good students to select dairying as a career.

R. H. LUSH has returned to the Tennessee campus from a two-year assignment to Brazil, as a pasture-forage specialist with the International Cooperation Administration. While in Brazil, Prof. Lush worked with Brazilian agricultural agencies, advising on research and extension work dealing with pasture, forage, and dairy cattle management.

The Univ. of Tennessee Dairy Institute was held Feb. 10-11 under the direction of the Dairy Dept. The Institute discussed topics in the

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fields of production, marketing, health, and quality. C. E. WYLIE, head of the Dairy Dept., reports that the University dairy staff was assisted by national authorities in each field of the program.

C. N. STARK, for many years a faculty member at Cornell Univ., Ithaca, N. Y., has joined the staff at the Univ. of Tennessee and has been assigned to work at Madras Univ., Madras, India.

In a letter to the Secretary-Treasurer of the A.D.S.A., Dr. Stark writes:

"Since I have been here, I have been made a professor of Madras Veterinary College, a professor of Madras Univ., and have been elected to a three-year term on the Academic Council of Madras Univ. Education in India is undergoing enormous changes. I hope I can have some influence on the changes. My title here is Dairy Production Advisor. At present I am directing the research of three men working for master's degrees in the dairy field . . . (the Indians) only want to be helped to help themselves. I am told that every fifth baby born into the world is an Indian. So many people can not be overlooked.

". . . I came here to make more and better milk available to the Indian people. Milk consumption in Madras (over 2,000,000 population) is said to be two ounces per person a day. Cows (and water buffalos) give 8 to 10 lb. of milk a day. The price of this milk must be high!"

Minnesota News

The FRANK ASTROTH Scholarship has been established at the Univ. of Minnesota, St. Paul, by Mrs. Astroth in memory of her late husband. It provides financial assistance for a student majoring in dairy production. The 1958-59 award was made to G. STRANGLAND, Mora.

C. KNODT has joined the staff of Cargill, Inc., as director of that company's new research farm at Elk River.

J. EDWARDS, manager of the production section of the Milk Marketing Board of England, addressed the dairy staff and students on the functioning of the Board in England, with special reference to herd improvement. He is an alumnus of the Univ. of Minnesota.

Dairy Industry Scholarships were awarded to ten young men who are working in the field of dairy industry at the University. These $300 scholarships are awarded on the basis of high scholastic standing and may be renewed from year to year, up to a total of $1,200 for four years. Recipients of the scholarships for this year are G. C. BEHRENS, Arlington, Ia.; D. J. BENNING, Browerville; R. R. DAY, Appleton; L. C. IVERSON, Mabel; H. D. JOHNSON,

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Theses Completed

Ph.D. Degree

J. L. ALBRIGHT—Spermatozoa survival of the bovine at 100° F., 40° F., and −110° F., as influenced by type and extent of dilution. State College of Washington, Pullman.

W. HAAB—The composition of fat triglycerides by countercurrent distribution. Univ. of California, Davis.

M.S. Degree

P. W. RIVERS—The influence of the type of activation on the fatty acids resulting from milk lipase action. Univ. of Minnesota, St. Paul.

C. M. STINE—A study of the lipid oxidation in dry whole milk. Univ. of Minnesota, St. Paul.

W. R. WINTERS—The use of contemporary herd averages in sire evaluation. State College of Washington, Pullman.

M. YAGUCHI—Vitamin B_{12} in cheese manufacturing. Univ. of California, Davis.

Raw Milk Pipeline Installed in France

Another pipeline installation will be made for transporting milk in the Haute Savois mountains in France to villages at the lower altitudes. The pipeline will be from 3 to 4 in. in diameter and will be placed 16 in. in the ground. Warm water will be flushed through for cleaning.

DAIRY JUDGING CHAMPS—The California State Polytechnic College, San Luis Obispo, dairy judging team won the 1957 Grand National Livestock Exposition championship from seven other western agricultural college teams. Along with the championship, they won a leg of the Bank of America high scoring team perpetual trophy (center). Pictured left to right are J. ALBRIGHT, dairy husbandry instructor and team coach; W. BARNES, R. BURK, R. THOMAS, and R. GOMES.

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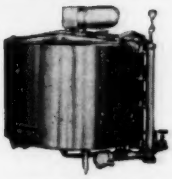

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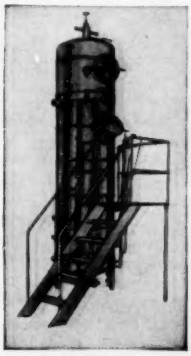

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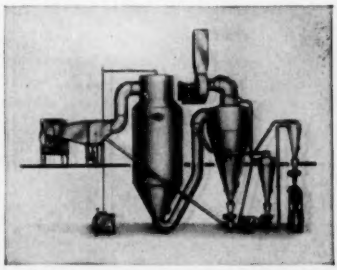

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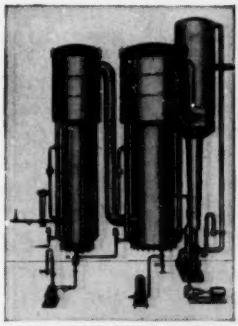

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FROM THE SECRETARY'S DESK

How Does One Attain a Responsible Position in the Dairy Industry?

Broadly speaking, to attain prominence in any industry, a man must have these qualifications: 1. Ability to get along with others. 2. Ability to plan. 3. Be a good speaker and writer. 4. Be ambitious. 5. Have experience. A college education is not mentioned, but it is obvious that it expedites the development of men for greater responsibilities. However, the college campus has no monopoly on American youth. A capable lad may excel the college graduate who does not have native ability. All men have these qualifications in varying degrees and many are able to earn college degrees. Nevertheless, the best-qualified persons are most likely to attain key positions in industry, but the percentage is small. It is frequently stated that fewer graduates in the Dairy Sciences attain responsibile positions than those majoring in Liberal Arts, the Sciences, or Engineering. This is owing to the fact that Dairy Science is highly specialized education, with only a few hundred graduates each year, whereas thousands graduate in other fields.

College staffs and industry executives feel that the number of students graduating in the Dairy Sciences should increase. It may be well to analyze the dairy industry for key opportunities, as compared to others. It consists of hundreds of small units. There are a few large companies such as Beatrice Foods, Borden's, Carnation, Foremost, and National Dairy Products, but the strength of all of them resides in a large number of relatively small plants. A dairy plant employing 200 people is the exception rather than the rule. Contrast this to the giants of the chemical, electrical, and automobile industries. It is obvious that these industries offer more opportunities in utilizing research and in engineering skills than are found in the dairy industry.

In the dairy industry, the most important positions, from the standpoint of responsibility and salary, are those of president, manager, sales manager, and plant superintendent. There are other key positions such as assistants to these executives who are engaged in procurement, engineering, quality control, office management, accounting, law, taxation, and labor relations.

It should be reemphasized that whereas there are opportunities for dairy technology graduates and those trained in the field of dairy science, the opportunities do not compare with those in other industrial fields. There is always

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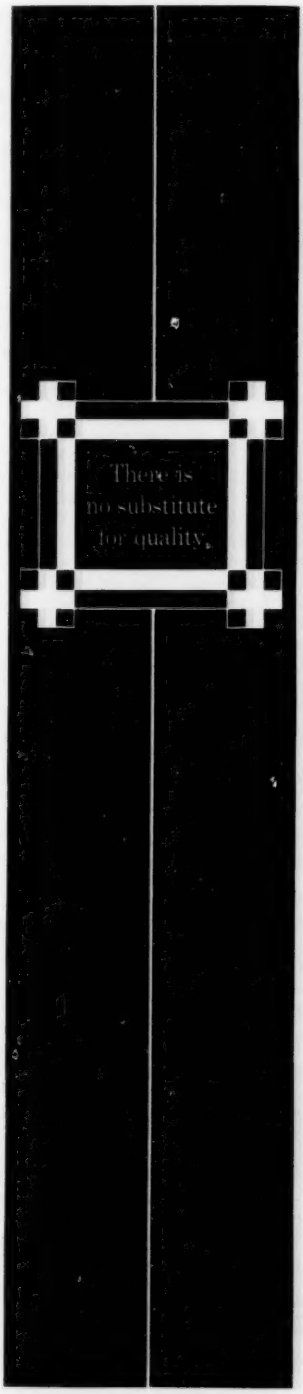

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2 The used disk is carefully removed from the strainer and placed on a piece of heavy paper. The producer then rinses the strainer, puts in a new *engineered* Rapid-Flo Single Faced Disk and proceeds with the next 4 cows, keeping track of which cows' milk is filtered through each disk.

3 After the foam disappears, each disk is examined. When he sees garget or foreign matter he filters the milk from each cow in that group individually at the next milking.

Examination of these disks will then indicate which cow, or cows, is causing trouble. This Rapid-Flo *every cow* Check-up will also indicate sources of extraneous matter and the steps necessary to produce clean milk.

Every producer can use this simple, common sense program to help avoid loss. When you recommend *engineered* Rapid-Flo Single Faced Disks and the Rapid-Flo Check-up you are helping him *see for himself* how to improve milk quality and profit.

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an opportunity for ownership of a dairy business, but most graduates do not have the money to establish a business in this very competitive field.

Another feature of the dairy business is that it is operated on smaller margins of profit as compared to other industries, and the salaries for responsible positions are lower. Naturally, this is the main reason why qualified young men major in science, engineering, or business administration. As long as there is a great demand for scientists and engineers, the dairy industry is not likely to be attactive, at present salary scales.

Dairy Technology graduates entering the dairy industry generally go into production or quality control. At present there is too great a spread in salaries between the production manager or plant superintendent and those of the sales or plant manager. Perhaps the job of production manager needs reappraising. The production manager, who has the responsibility of turning out the product, has many duties and little assistance. The result is that in many plants operating efficiency is unknown. A production manager should have time to study plant operations and work out plans to improve efficiency. Suppose, for example, that all losses in a plant making two million gallons of ice cream, amount to 3 cents a gallon, or $60,000 a year. Let's further assume that a good production man reduces this loss to ¼ cent a gallon. What is this man worth? The salary level for competent production managers should be raised, because increased profits must come, mostly, from more efficient methods. Operating a plant, handling labor, and selling executives productive ideas and putting them into practice, are the manager's responsibility.

Most dairy plants do not have adequate programs for training young men for key positions, while other industries do have such programs. Graduates should get most of their practical training in industry, because the colleges need four years to train them in fundamentals. In college, the student should get a broad fundamental education, besides a training in the dairy field.

A committee in the American Dairy Science Association is developing a training manual for the dairy industry. It may help, provided students have a good fundamental training.

It is a pleasure to state that the dairy industry has some advantages over other industries. It is stable. During the depression of the late twenties and early thirties, employment in the dairy industry was 80% of normal, as compared to much lower levels in other industries. There is a humanitarian side, too—dairy products are needed from the cradle to the grave. The industry is widespread and found everywhere in the civilized world. After 30 years in the dairy industry, prior to retirement, the writer would do the same thing again.

H. F. JUDKINS

A COMPARISON OF SOME OF THE CHEMICAL AND PHYSICAL PROPERTIES OF γ-CASEIN AND IMMUNE GLOBULINS OF MILK[1]

G. K. MURTHY[2] AND R. McL. WHITNEY

Department of Food Technology, University of Illinois, Urbana

SUMMARY

To identify the slowest component in the electrophoretic pattern of skimmilk at pH 8.7 and to investigate further the chemical and physical properties of γ-casein and the immune globulins, these proteins were isolated from milk and colostrum. Phosphorus analyses indicate a distinct difference between γ-casein and immune globulins. In veronal buffer at pH 8.7, $\mu = 0.1$, the electrophoretic mobilities of γ-casein and pseudoglobulin are the same. In glycine hydrochloride buffer pH 2.3, $\mu = 0.1$, the three proteins have different electrophoretic mobilities, but a mixture of γ-casein-euglobulin interacts, the extent depending on the time of storage at 4° C. While γ-casein is electrophoretically homogeneous in glycine hydrochloride buffer at pH 2.3 and 3.25 and $\mu = 0.1$, it is heterogeneous in comparable sodium lactate buffers, due to interactions with the lactate ion. All three proteins contribute to the area of the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.7. They are heterogeneous in the ultracentrifuge. The molecular weight calculated for the major components of γ-casein varied with pH; whereas, the frictional ratios were the same in veronal and glycine hydrochloride buffers but decreased in sodium lactate buffer. The molecular weights of euglobulin and pseudoglobulin were not pH-dependent, but their frictional ratios increased with decreasing pH values.

Recently, the Protein Committee *(11)* of the American Dairy Science Association has clarified the current nomenclature for the milk proteins. They have recognized, on the basis of the work of McMeekin *(15)*, that the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.4–8.7 and 0.1 ionic strength is the γ-casein. However, Heyndrickx and DeVleeschauwer *(6, 7)* neither recognized nor reported the presence of γ-casein, and they have attributed a globulin character to the same component, both in colostrum and in milk. Comparison of some of the properties of γ-casein and immune globulins available in the literature *(4, 8, 21, 22)* shows that these proteins have approximately the same isoelectric point, electrophoretic mobility at pH 8.7, nitrogen, sulfur, lysine, and valine contents. In order to clearly identify the slowest-moving component in the electrophoretic pattern of skimmilk at pH 8.7, and further to compare these proteins, γ-casein and the immune globulins were isolated from milk and colostrum, respectively, and some of their chemical and physical properties were studied.

Received for publication August 23, 1957.

[1] Taken from the thesis of G. K. Murthy, presented in June, 1956, to the University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

MATERIALS AND METHODS

γ-Casein. Casein was prepared according to Cherbuliez and Baudet *(2)* from selected Holstein cows' milk and fractionated to obtain γ-casein, by the method described by Hipp *et al. (8)*.

Immune globulins. Euglobulin and pseudoglobulin were prepared from colostrum, first milking after parturition, by the method described by Smith *(21)* for their isolation from bovine whey.

Phosphorus determination. Phosphorus contents of the proteins were determined using Verma's *(24)* technique for digesting the samples, and Fiske and Subbarow's *(3)* method for color development. The blue color developed was measured in a Coleman Model 11 Spectrophotometer at 660 mμ.

Electrophoretic analyses. Electrophoretic analyses of the isolated proteins were performed at ice bath temperatures in sodium veronal (pH 8.7, $\mu = 0.1$), glycine hydrochloride (pH 2.3, $\mu = 0.1$), and sodium lactate (pH 2.3, $\mu = 0.1$) buffers. One per cent protein suspensions were prepared either singly or in mixtures, in the ratio of 1:1. Since the electrophoretic pattern of γ-casein-euglobulin mixture obtained at pH 2.3 was unexpected, the analysis was repeated after 24 hr. of storage of the mixture at 4° C.

Since the electrophoretic patterns of γ-casein in lactate buffer at pH 2.3 were heterogeneous, possibly due to the low buffer capacity of this buffer at this pH, a special comparison was made of the electrophoretic pattern of γ-casein in glycine and lactate buffers at pH 3.25 and $\mu = 0.1$, where the buffer capacities of the two systems were the same.

In order to confirm the identity of the slow-moving component of skimmilk at pH 8.7, γ-casein, euglobulin, and pseudoglobulin each were added, at 0.1% concentration, to a portion of skimmilk diluted 1:2.5 with sodium veronal buffer, assuming the protein concentration of the diluted skimmilk to be 1.0%. These systems were then electrophoretically analyzed in the usual manner.

Diffusion analyses. Diffusion analyses of most of the same protein suspensions prepared for electrophoresis were performed at 2° C. in a Tiselius electrophoresis apparatus. The initial boundaries were created according to the method described by Lundgren and Ward *(14)*, and the diffusion constant was calculated by the area and height method. The values were corrected to standard conditions in the usual manner.

Viscosity. The viscosity measurements were made with the Höeppler viscosimeter *(10)*. In order to avoid the use of instrument constants which would change with temperature, the viscosity of the buffers was calculated by comparison with water over the temperature range of from 0 to 10° C. The viscosity of water at the various temperatures was obtained from Lange *(12)*.

Density. The densities of the buffers at the various temperatures were determined with the Westphal balance.

Sedimentation analyses. Sedimentation analyses were carried out at from 4 to 6° C. in a Spinco Model E Ultracentrifuge, with 1.0 and 0.5% protein suspensions in sodium veronal and glycine hydrochloride buffers. For compari-

son purposes, γ-casein was analyzed at 0.5% protein in sodium lactate and glycine hydrochloride buffers of pH 2.3 and pH 3.25, and $\mu = 0.1$. The observed sedimentation constants were corrected to standard conditions in the usual manner. The partial specific volumes were obtained from the literature *(15, 21)*. The $S_{20,w}$ values were extrapolated to zero protein concentration and the extrapolated values were used in the calculation of apparent molecular weight.

RESULTS AND DISCUSSION

The phosphorus content of γ-casein (0.107%) is in close agreement with Hipp's value—0.11% *(8)*. Analyses of the immune globulins support the findings of Smith *(20, 21)*, in that they do not indicate the presence of any phosphorus; therefore, the globulins in this respect show a marked difference from γ-casein.

Electrophoretic analyses. Results of the electrophoretic analyses are presented (Table 1). The isolated γ-casein contained from 5 to 8% of a slow-moving component which might be the δ-casein, as suggested by Cherbuliez and Baudet *(2)*. In veronal buffer, the mobility of γ-casein conforms with Hipp's *(8)* value, -2.01×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}. The mobility of euglobulin is approximately the same as that observed by Smith *(22)*, -1.8×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}; whereas, that of pseudoglobulin is different from his value of -2.2×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}. The γ-casein and pseudoglobulin have practically the same mobility

TABLE 1

Electrophoretic properties of γ-casein and immune globulins

Protein	Descending		Ascending	
	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)
		Veronal buffer, pH 8.7, $\mu = 0.1$, 1% protein		
γ-Casein	100	−2.03	100	−2.32
Euglobulin	100	−1.76	100	−1.81
Pseudoglobulin	100	−2.04	100	−2.13
γ-Casein + euglobulin (1:1)	53.3	−1.74	51.8	−1.85
	46.7	−2.03	48.2	−2.32
γ-Casein + pseudoglobulin (1:1)	100	−2.00	100	−2.30
		Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$, 1% protein		
γ-Casein	100	3.23	100	4.33
Euglobulin	100	5.28	100	6.12
Pseudoglobulin	100	5.17	100	5.99
γ-Casein + euglobulin (1:1)	13.9	2.14	53.6	4.41
44-hr. dialysis	51.4	3.57	46.4	5.97
	34.7	5.19		
γ-Casein + euglobulin (1:1)	27.1	3.05	56.4	4.54
44-hr. dialysis +	45.0	4.09	43.6	5.97
24-hr. storage	27.8	5.19		
γ-Casein + pseudoglobulin (1:1)	50	3.39	49.1	4.32
	50	5.12	50.9	6.01

and in mixtures move with a single maximum. In mixtures, γ-casein and euglobulin move independently with the same mobility as they possess in the isolated systems and, therefore, indicate no complex formation between them.

In glycine hydrochloride buffer, the patterns of γ-casein and immune globulins are sharper than in veronal buffer. While the γ-casein and pseudoglobulin move independently in mixtures showing no complex formation between them, the γ-casein-euglobulin mixture resulted in three components on the descending side (Figure 1), the faster component having the mobility of euglobulin and the

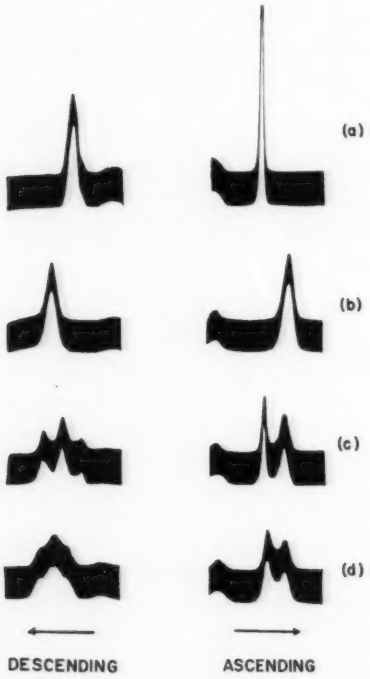

FIG. 1. Electrophoretic patterns obtained in a glycine-HCl buffer (pH 2.3 ionic strength of 0.1) with a protein concentration of 1.0%, after electrophoresis for 4,500 sec. *(a)* γ-casein at field strength of 9.35 V/cm; *(b)* euglobulin at field strength of 9.16 V/cm; *(c)* γ-casein-euglobulin (44-hr. dialysis) at field strength of 9.09 V/cm; *(d)* γ-casein-euglobulin (44-hr. dialysis, 24 hr. of storage) at field strength of 9.09 V/cm.

other two components differing in mobility, one faster and the other slower than γ-casein. On the ascending side, only two components are observed, with the faster component having the same mobility as euglobulin and the slower component moving faster than γ-casein. To confirm this unexpected result, the electrophoretic analysis was repeated after 24 hr. of storage at 4° C., and a similar result was obtained; however, the relative areas of the various components on the

descending side were found to have changed (Figure 1), along with their mobilities, except for the mobility of the fastest component, euglobulin. These results indicate the possibility of a complex formation similar to that observed by Chargaff *et al. (1)*, in their electrophoretic studies of serum albumin-heparin mixtures, and by Longsworth and McInnes *(13)*, with mixtures of ovomucoid and nucleic acid. Since there was a change in the electrophoretic pattern with time of storage, the rate of attainment of equilibrium is very slow when compared to electrophoretic separation. However, the association observed cannot be explained from the interaction types proposed by Longsworth, since, in this case, the velocity constant for the forward reaction, k_1, and for the reverse reaction, k_2, are both small, but k_1 is greater than k_2.

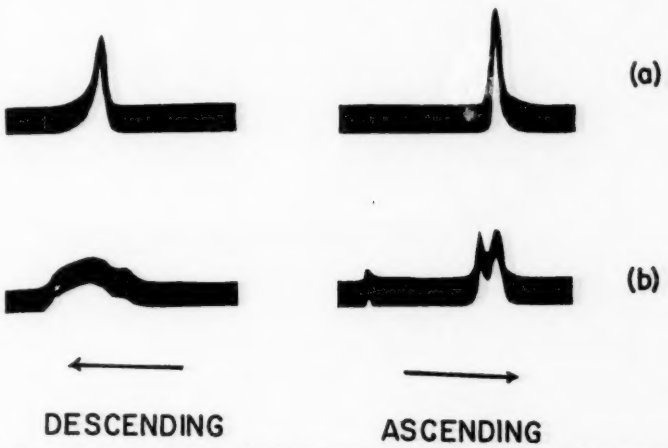

FIG. 2. Electrophoretic patterns obtained in a glycine-HCl buffer and a lactate buffer (pH 3.25 ionic strength of 0.1) with a protein concentration of 0.5%, after electrophoresis for 5,400 sec. *(a)* γ-casein in glycine-HCl buffer at field strength of 10.69 V/cm; *(b)* γ-casein in lactate buffer at field strength of 12.62 V/cm.

γ-Casein is electrophoretically homogeneous in glycine hydrochloride buffer of pH 2.3 and 3.25, and $\mu = 0.1$, and it is heterogeneous in the comparable sodium lactate buffers (Figure 2). This behavior of γ-casein in lactate buffer is similar to that observed by Hipp *et al. (8)*. The patterns obtained at pH 3.25 show that this heterogeneity is not due to a low buffer capacity but to an interaction with the buffer ion.

The electrophoretic properties (Table 2) of the isolated γ-casein, euglobulin, and pseudoglobulin, upon their addition to skimmilk, show that these proteins, irrespective of their mobilities in the isolated system, behave similarly. In skimmilk, they all appear as the slow-moving component, with approximately the same mobility of -2.01×10^5 cm.^{2} sec.$^{-1}$ volt^{-1}.

Diffusion. The observed diffusion constants, $D_{z,b}$ (Table 3), in veronal buffer for the immune globulins, are approximately the same as those reported

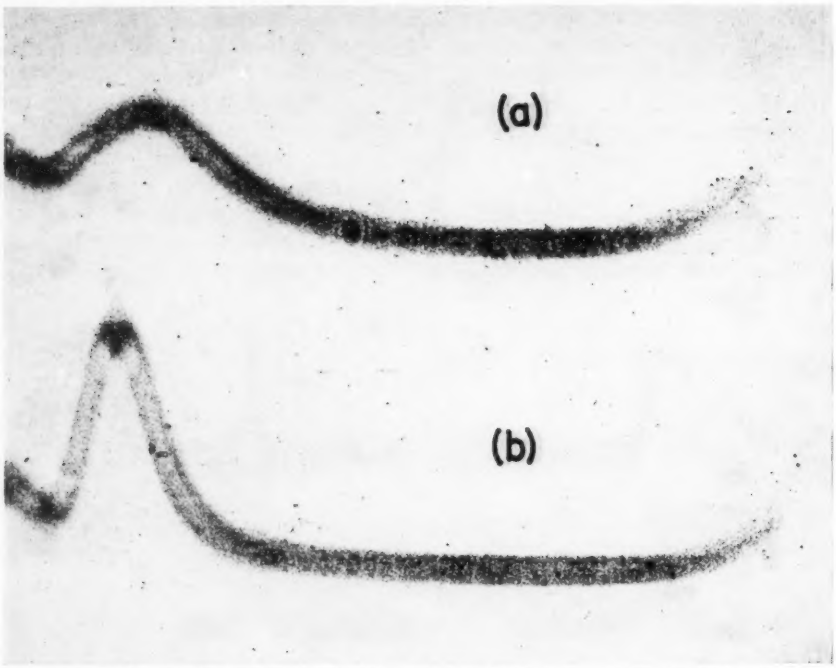

FIG. 3. Sedimentation pattern obtained in a glycine-HCl buffer and lactate buffer (pH 3.25, ionic strength of 0.1) with a protein concentration of 0.5% at 12.5° C. *(a)* γ-casein in glycine-HCl buffer, *(b)* γ-casein in lactate buffer.

by Smith *(21)*. However, the diffusion constants calculated at standard conditions are lower. This may be owing to the differences in the measured viscosities of the buffer and the methods employed in their determination. The diffusion constant of γ-casein is considerably higher than that of the immune globulins. The γ-casein-pseudoglobulin mixture diffuses at a slightly faster rate than the average of their diffusion constants. The relative areas under the diffusion curves of the mixtures, γ-casein, and pseudoglobulin are significantly different; therefore, the rate of change of index of refraction with concentration of the individual components must be different in mixtures than it is in the individual systems. Hence, the slightly faster than average diffusion rate of the mixture may not indicate any interaction between them, since Gosting *et al. (5)* show that the diffusion constant of a mixture in which there is no interaction need not be the weighted average of the diffusion constants of the components, provided the rates of change of index of refraction with concentration for the individual components are different. In glycine hydrochloride buffer, the diffusion constants of all three proteins are lower than in veronal buffer, with the euglobulin and pseudoglobulin diffusing at the same rate. The diffusion constant of the γ-casein and euglobulin mixture is as expected for a 50:50

TABLE 2

Electrophoretic properties of isolated γ-casein and immune globulins upon their addition to skimmilk [a]

Sample	α-Casein		β-Lactoglobulin		β-Casein		Component 4	
	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	Relative area (%)	Mobility (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)
				Descending				
Skimmilk	69.3	−5.61	5.2	−4.59	24.4	−3.17	1.0	−1.98
Skimmilk + γ-casein	64.6	−5.51	6.1	−4.59	21.3	−3.17	8.0	−2.02
Skimmilk + euglobulin	65.0	−5.61	6.4	−4.59	21.5	−3.17	7.1	−2.02
Skimmilk + pseudoglobulin	64.5	−5.40	6.1	−4.50	21.6	−3.05	7.6	−2.05
				Ascending				
Skimmilk	61.8	−6.43	7.4	−5.56	29.2	−4.00	1.6	−2.80
Skimmilk + γ-casein	57.9	−6.34	7.1	−5.42	26.0	−3.95	9.0	−2.78
Skimmilk + euglobulin	58.0	−6.43	7.3	−5.42	25.4	−3.95	9.2	−2.66
Skimmilk + pseudoglobulin	57.2	−6.34	7.9	−5.42	25.6	−3.91	9.3	−2.66

[a] Electrophoresis was performed at ice-bath temperatures in veronal buffer, pH 8.7, $\mu = 0.1$.

TABLE 3

Diffusion constants of γ-casein and immune globulins

	Diffusion constant	
	$D_{2,b}$	$D_{20,w}$
	(cm.² sec.⁻¹ × 10⁷)	
	Veronal buffer, pH 8.7, $\mu = 0.1$, 1% protein	
γ-Casein	2.71 ± 0.06	4.82
Euglobulin	1.63 ± 0.04	2.91
Pseudoglobulin	1.82 ± 0.04	3.23
γ-Casein + pseudoglobulin (1:1)	2.40 ± 0.06	4.26
	Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$, 1% protein	
γ-Casein	1.13 ± 0.10	1.87
Euglobulin	1.59 ± 0.04	2.60
Pseudoglobulin	1.59 ± 0.04	2.60
γ-Casein + euglobulin (1:1)	1.30 ± 0.05	2.16
	Lactate buffer, pH 2.3, $\mu = 0.1$, 1% protein	
γ-Casein	1.13 ± 0.03	2.18

mixture, assuming no interaction; however, the observed value must be the average of the various species of γ-casein and euglobulin present. In sodium lactate buffer (pH 2.3), γ-casein diffuses slightly faster than in glycine hydrochloride buffer (pH 2.3).

Sedimentation. As expected, the rate of sedimentation of γ-casein, euglobulin, and pseudoglobulin are concentration-dependent, increasing with decreasing protein concentration. In veronal buffer, γ-casein has a low $[S_{20,w}]_{c=o}$ value of 1.55 Svedberg units, and sediments with a single, although broad, maximum. Prolongation of sedimentation time during the analysis did not reveal any additional maxima, but the spreading of the peak is greater than predicted by diffusion. However, in glycine hydrochloride buffer, γ-casein possesses at least three components sedimenting close to one another, the major component, 67%, having a $[S_{20,w}]_{c=o}$ value of 10.30 Svedberg units. In lactate buffer (pH 2.3, and 3.25, $\mu = 0.01$) it is more homogeneous. McMeekin and Peterson (16) observed that γ-casein is heterogeneous in the ultracentrifuge (the rates of sedimentation of the components depending upon the temperature of analysis) and that the system behaves reversibly. It appears from this study that the sedimentation of γ-casein depends also on the pH and the type of buffer used. The euglobulin and pseudoglobulin are heterogeneous both in veronal and glycine hydrochloride buffers. The euglobulin yielded three components with the major component, from 86 to 87% of the total area; whereas, the pseudoglobulin yielded two components with the major component, from 84 to 89.3% of the total area. These results are in good agreement with the sedimentation constants obtained by Smith and Brown *(23)* in 0.15 *M* NaCl at room temperature.

Molecular weights. Although the heterogeneity of these proteins is readily apparent, it is interesting to calculate the apparent molecular weights of

TABLE 4

Sedimentation constants of γ-casein and immune globulins

Protein	Sedimentation constant ($S_{20,w}$)		
	Protein		
	1.0%	0.5%	0.0%
	Veronal buffer, pH 8.7, $\mu = 0.1$		
γ-Casein	1.41 (100%)	1.49	1.55
Euglobulin	7.93 (87.0%)	8.36	8.77
	11.85 (10.0%)	11.81	11.7
	22.98 (3.0%)		22.0
Pseudoglobulin	7.69 (89.3%)	7.91	8.07
	10.7 (10.7%)	10.94	11.17
	Glycerine hydrochloride buffer, pH 2.3, $\mu = 0.1$		
γ-Casein	— (15.0%)	—	—
	9.72 (67.0%)	10.03	10.30
	— (18.0%)	—	—
Euglobulin	6.91 (86.0%)	7.33	7.75
	10.51 (11.0%)	—	—
	— (3.0%)	—	—
Pseudoglobulin	7.04 (84.0%)	7.10	7.18
	10.21 (12.0%)	10.30	10.39
	— (4.0%)	—	—
	Glycerine hydrochloride buffer, pH 3.25, $\mu = 0.1$		
γ-Casein		—	
		17.39	
	Lactate buffer, pH 2.3, $\mu = 0.1$		
γ-Casein		14.54 (100%)	
	Lactate buffer, pH 3.25, $\mu = 0.1$		
γ-Casein		11.48 (100%)	

the major components of euglobulin and pseudoglobulin from sedimentation and diffusion constants $[S_{20,w}]_{c=0}$ and $[D_{20,w}]_{c=1.0\%}$ (Table 3). The molecular weights of each are approximately the same, either in veronal or glycine hydrochloride buffer, with an average value of 252,000 and 289,000, respectively. Smith *(22)* reported the molecular weights of these immune globulins (milk and colostrum) to be in the neighborhood of from 160,000 to 190,000. This discrepancy may be owing to the methods of selection of the values for diffusion and sedimentation constants to be used in Svedberg's formula. He assumed a value for the diffusion constant greater than 3.9×10^{-7} cm.^{2} sec^{-1}, although his values for the immune globulin varied between 3.24 and 3.52×10^{-7} cm.^{2} sec.$^{-1}$ for bovine whey and 3.86×10^{-7} cm.^{2} sec.$^{-1}$ for colostrum pseudoglobulin, and he gave a value of 7.0 Svedberg units for their sedimentation constant. It is not clear whether or not the sedimentation constant used was obtained by extrapolation to zero protein concentration.

The molecular weights of γ-casein (Table 5) show large differences as a result of environment, indicating association of the protein molecule at low pH values.

Frictional ratios. As in the case of the calculation of molecular weights, the heterogeneity of the protein raises some question as to the validity of calculated frictional ratios. However, the molar frictional ratios (f/f_o), for

TABLE 5

Molecular weight of major component of γ-casein and immune globulins

Protein	Molecular weight		
	Veronal buffer (pH 8.7, $\mu = 0.1$)	Glycine HCl buffer (pH 2.3, $\mu = 0.1$)	Lactate buffer (pH 2.3, $\mu = 0.1$)
γ-Casein	30,650	537,000	659,000
Euglobulin	291,000	287,000	
Pseudoglobulin	243,000	262,000	

the immune globulins (Table 6) are greater than unity and, therefore, indicate that these protein molecules are either hydrated or asymmetrical. However, when the values for (f/f_o) are expressed as products of hydration (f/f_e) and asymmetry (f_e/f_o), assuming reasonable values for hydration, the results can not be explained on the basis of hydration alone. The axial ratios calculated using Perrin's *(18)* equation are higher at pH 2.3 than at pH 8.7, although the molecular weights are approximately the same at both the pH levels. It is possible to explain these observations on the basis of changes in asymmetry with pH. Polson *(19)* and Neurath *(17)* have calculated the minimum value for the radius of minor axis of several proteins to be from 8 to 9 Å. At pH 8.7, the calculated radius of the minor axis at constant hydration (0.2 g. bound water per gram of protein) can be approximated if the cross section is assumed to consist of four protein units of radius 8.5 Å; whereas, at pH 2.3, it can be approximated with three protein units in the cross section of the molecule. On the other hand, at a constant value for the axial ratios, the changes in the frictional ratios can be explained on the basis of a change in hydration only in the case of euglobulin. For pseudoglobulin, it would be necessary to assume unreasonable values for hydration.

For γ-casein, the observed axial ratios calculated for an elongated ellipsoid differ markedly from those reported by Hipp *et al. (9)*, who obtained values

TABLE 6

Frictional ratios and dimenions of γ-casein and immune globulins

Protein		Hydrated (0.2 g. H_2O/g protein)			Hydrated (0.8 g. H_2O/g protein)		
			Axial ratio	b		Axial ratio	b
	f/f_o	f_e/f_o	(a/b)	$(Å)$	f_e/f_o	(a/b)	$(Å)$
			Veronal buffer, pH 8.7, $\mu = 0.1$				
γ-Casein	2.080	1.944	19.4	8.5	1.687	12.9	9.12
Euglobulin	1.648	1.526	9.6	20.9	1.308	5.9	24.40
Pseudoglobulin	1.587	1.469	8.6	20.3	1.260	5.5	23.60
			Glycine hydrochloride buffer, pH 2.3, $\mu = 0.1$				
γ-Casein	2.100	1.944	19.4	20.2	1.667	12.4	23.20
Euglobulin	1.846	1.709	13.2	18.6	1.465	8.5	21.50
Pseudoglobulin	1.895	1.754	14.3	17.5	1.504	9.2	20.40
			Lactate buffer, pH 2.3, $\mu = 0.1$				
γ-Casein	1.710	1.600	11.0	26.0	1.357	6.7	30.70

of 9.3 for the elongated and 16.2 for the prolate molecule, based upon the intrinsic viscosity measurements in NaOH–NaCl system of pH 7.2 and $\mu = 0.05$. The lack of agreement is undoubtedly due to the differences in solvent systems and the methods of measurement. No difference in the axial ratios in veronal and glycine hydrochloride buffers is observed. The observed value of 8.5 Å for the radius of the minor axis of γ-casein is in close agreement with the minimum values of from 8 to 9 Å calculated by Polson *(19)* and Neurath *(17)*. However, the molecular weight in glycine hydrochloride buffer is approximately 18 times larger than that in veronal buffer. To account for the increase in molecular weight without appreciable change in the axial ratios, we can postulate an aggregation of 18 of the molecular species present at pH 8.7, with a maximum cross section of four of them. Such a molecule at 0.2 g. of bound water per gram of protein would have a radius of 19.6 Å, compared with the calculated radius of 20.2 Å.

The molecular weight in lactate buffer is approximately 22 times larger than in veronal buffer, with appreciable changes in frictional ratios. These differences can be explained by assuming an aggregation of 22 of the molecular species present at pH 8.7 and at 0.8 g. of bound water per gram of protein, with a maximum cross section of eight of them. Such a molecule will have a radius of 30.0 Å, compared with the calculated radius of 30.7 Å.

In conclusion, the above observations show: that γ-casein and immune globulins, although different proteins, are all components of the slow-moving peak in the electrophoretic pattern of skimmilk at pH 8.7; the molecular weights of the immune globulins are different from those reported by Smith, and the molecular weight of γ-casein is a function of pH and buffer ion as well as temperature; whereas, the hydration and/or asymmetry of all the proteins studied are pH-dependent.

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SEPARATION, CONCENTRATION, AND PROPERTIES OF ALPHA-AMYLASE FROM COWS' MILK[1]

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SUMMARY

The presence in milk of α-amylase was confirmed. Analyses for the enzyme in milk and fractions thereof were made by measuring the rate of decrease in viscosity of starch paste with Ostwald viscosimeters. Analysis of milk and that dialyzed with various added salts revealed that the enzyme requires both calcium and chloride for activity. When milk proteins were fractionated, much of the α-amylase activity was concentrated into the lactoglobulin fraction precipitated from whey at 43% saturation with $(NH_4)_2SO_4$. The enzyme was further concentrated by dissolving the lactoglobulin in a solvent consisting of ethylene glycol, ethanol, and water in the ratio of 15:29:56 by volume, adsorbing it upon rice starch and eluting with saturated $CaSO_4$ solution. A preparation with 30 times the specific activity of lactoglobulin and containing 20% of the total activity of the lactoglobulin from which it was prepared, had starch liquefying, dextrinizing, and saccharifying properties. The optimum pH for the enzyme was 7.4 at 34° C. The energy of activation of the catalyzed reaction, measured by starch liquefaction, was 8,500 calories per mole. The enzyme was progressively inactivated by 30-min. heating between 45 and 52° C. at pH 6.4. A plot of velocity vs. velocity/substrate concentration was linear, indicating a single enzyme entity.

Alpha-amylases hydrolyze starch and glycogen by splitting central α-1-4-D-glucosidic linkages, causing rapid liquefaction and slower dextrinization and saccharification. They are found in plants, microorganisms, and in the pancreas, blood, urine, and saliva of animals *(8)*. They usually require either calcium or chloride ions. On the other hand, β-amylases cause rapid saccharification of starch with slow liquefaction, by splitting off terminal maltose units. They have been positively identified only in plants. They require no ionic activators but require free -SH groups for activity.

The presence of α-amylase in normal cows' milk has been reported by Sato *(12)*, Chrzaszcz and Goralowna *(1)*, and Richardson and Hankinson *(11)*. Gould *(2)* used α-amylase in cows' milk as an index of the efficiency of pasteurization. Schloemer *(13)* found that both Ca^{++} and Cl^- were necessary for maximum activation of the enzyme in dialyzed lead sera from milk. All of these authors measured α-amylase activity by the Wohlgemuth *(17)* starch-iodine method, which is a measure of dextrinization, but Richardson and Hankinson *(11)* also demonstrated liquefaction by viscosity measurements, and noted an increase in reducing sugars when starch and milk were incubated together for 24 hr. They attributed this saccharifying power to a separate enzyme (β-amylase), since it persisted in milks heated at 65° C. for 30 min.; whereas, the loss in starch-iodine color was not observed in milks heated at 50° C. or over

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for 30 min. Inasmuch as α-amylase also produces free sugar, it is difficult to explain the observation of Richardson and Hankinson, that the saccharifying power was not diminished at all by heating the milk sufficiently to inactivate the dextrinifying power completely (55° C. for 30 min.). Schloemer *(14)* was unable to detect the presence of β-amylase in cows' milk, finding only a zone corresponding to α-amylase when a lead serum from milk was allowed to diffuse into a starch-containing gelatin gel which was subsequently stained with iodine.

Since the physical and chemical properties of the α-amylase of milk are not well defined, a more detailed study of this enzyme was undertaken. For this purpose, the enzyme was concentrated by fractionation of the milk proteins.

MATERIALS AND METHODS

Starch substrate. Raw white potato starch was obtained from a lot prepared by Loewus *(7)*. It contained 14.7% moisture (determined by vacuum oven drying at 105° C.). From this a standard stock starch paste containing 4.25% starch (wet weight basis) was prepared, according to a modification (no buffers added) of the method of Landis and Redfern *(6)*.

Milk source. All milk was obtained from the University herd or from the Twin City Milk Producers' Association. Samples used for fractionation were processed immediately upon receipt. Milk samples for assay were sometimes stored under toluene at 3° C. for periods up to a week.

Procedure for measuring liquefaction. Alpha-amylase activity was measured primarily by the method of Hultin *(5)*, in which the activity per unit of enzyme-containing material is calculated from the rate of change of the reciprocal of the specific viscosity as follows:

$$\mu\text{A/g or } \mu\text{A/ml} = \frac{d\eta_{sp}^{-1}}{dt} \times C^2 \times 10^6 \times \frac{V}{a} \qquad (1)$$

where: μA = microactivity unit

η_{sp} = specific viscosity = $\eta_r - 1$

t = time (Hultin used seconds, but in this paper the time was taken in minutes. Thus, the units reported here are 1/60th of Hultin's)

C = concentration of starch (14.7% moisture basis) in the reaction mixture in grams per ml.

a = amount of enzyme solution (grams or ml.) in the reaction mixture

V = total grams or ml. of reaction mixture.

Viscosities were measured with Ostwald viscosimeters having outflow times varying from 34.1 to 42.1 sec. with 0.025 N acetic acid at 30.0° C. Reaction mix-

tures consisted of 0.5 to 1.5 ml. of the solution being assayed, 7.1 g. of starch paste (previously tempered to the reaction temperature), and buffer and activators sufficient to make 10 ml. Thus, the initial starch concentration of the reaction mixture was approximately 3% (wet weight basis.) Exactly 8 ml. of reaction mixture was pipetted into the viscometer and incubated therein at either 34° C. ± 0.1° C. or 48° C. ± 0.1° C. Four or five outflow times were determined in seconds at intervals during a total elapsed time of from 25 to 40 min. Specific viscosities were calculated as follows:

$$\eta_{sp} = \frac{t_s - t_o}{t_o}$$

where: t_s = outflow time for assay solution

t_o = outflow time for 3% starch paste fully liquefied by concentrated bacterial amylase

The reciprocal of specific viscosity, η_{sp}^{-1} was then plotted against incubation time; in all cases such plots were linear. The slope of the plot $\frac{d\eta_{sp}^{-1}}{dt}$ was calculated and corrected by subtraction of the slope obtained with blanks containing boiled enzyme solution or water, instead of active enzyme. The corrected slope value was used to calculate activity ($\mu A/g$ or $\mu A/ml$) according to Equation (1). In general, the rate of decrease of viscosity of the blank did not amount to more than from 2 to 5% of that of the test sample.

Procedures for measuring saccharification. Sugar liberated from the starch substrate was measured by the dinitrosalicylic acid method of Sumner and Howell *(16)*, and with picric acid by a modification of the method of Perry and Doan *(10)*. Standard curves for each method were made with maltose monohydrate. With either method, reducing sugar equivalent to from 0.2 to 1.0 mgm. of maltose could be determined.

The dinitrosalicylic acid method was used only with purified serum proteins. Test solutions were made with a total volume of 10 ml. of starch, buffer, and enzyme (and activators when used). Specific details of the composition of the reaction mixture, and the incubation conditions, are given in connection with individual experiments. At intervals during incubation, 1-ml. aliquots were withdrawn and added to 5 ml. of dinitrosalicylic acid reagent and 1 ml. of 0.25 *N* NaOH. This mixture was heated for 6 min. in a boiling water bath and cooled. Ten ml. of water was added and absorbance read at 540 mμ with a Coleman Model 11 Spectrophotometer. Corrections were made for the influence of such activators as cysteine hydrochloride and $CaCl_2$ on the color development. Blanks were made by separately adding the buffer, starch, and enzyme to 5 ml. of the dinitrosalicylic acid, then heating and determining absorbance as with the samples.

The picric acid method was used to measure reducing sugar produced by dialyzed skimmilk and in some cases by protein fractions. Typical test solutions consisted of 4 ml. of dialyzed milk, 5 ml. of 4.25% starch, and 1 ml. of buffer.

After incubation, exactly 2 ml. of this solution were added to 2 ml. of saturated picric acid, causing precipitation of the milk proteins. The mixture was filtered through glass wool and 2 ml. of the filtrate added to 2 ml. of 25% Na_2CO_3 and an additional 1 ml. of saturated picric acid. This mixture was heated for 20 min. in a boiling water bath, made to 10 ml. with distilled water, and the absorbance read at 520 mμ.

TABLE 1

Starch liquefying power of milk and dialyzed milk

Lot	Liquefying power[a]	
	Not dialyzed	Dialyzed
	($\mu A/ml$)	
1. Whole milk–herd	10.4	26.7
2. Whole milk–herd	20.4	31.3
3. Whole milk–commercial	13.5	13.7
Skimmilk–commercial	14.1	15.6
4. Skimmilk–commercial	14.1	16.2
5. Whole milk–commercial	17.4	17.4
Skimmilk–commercial	19.8	23.6
6. Whole milk–commercial	15.6	18.8
Skimmilk–commercial	16.7	19.9
7. Skimmilk–commercial	15.0	16.4

[a] All determined in presence of 0.06 or 0.075 *M* $CaCl_2$, 34° C., pH 7.3–7.4 (THMAM buffer), 3.0% starch substrate.

Procedure for measuring dextrinization. The dextrinization produced by purified α-amylase was measured by a modification of the method of Smith and Roe *(15)*, in which the concentration of starch remaining at any time is determined colorimetrically by its reaction with iodine. The specific conditions used were: pH 7.40, tris (hydroxymethyl) aminomethane (THMAM) buffer of ionic strength 0.035, 0.075 *M* $CaCl_2$, 34° C., and 0.6% starch concentration. Dextrinizing power was expressed in terms of mgm. of starch hydrolyzed in 30 min., calculated as follows:

$$\text{Starch hydrolyzed in 30 min. (mgm.)} = \left(\frac{A_{\text{control}} - A_{30\text{ min.}}}{A_{\text{control}}} \right) \times 60$$

where: A_{control} = absorbance at 620 mμ for the control containing 60 mgm. starch per tube

$A_{30\text{ min.}}$ = absorbance at 620 mμ for sample incubated for 30 min.

Procedure for measuring protein content. The protein content of various fractions was routinely followed by a turbidimetric method using trichloroacetic acid as a protein precipitant. Three ml. of 50% trichloroacetic acid were added to 5-ml. aliquots of diluted protein solution in matched colorimetric tubes. At the end of 5 to 10 min., absorbance readings were taken on a Coleman 11 Spectrophotometer at 650 mμ. A standard curve was constructed with lactoglobulin relating mgm. of protein N (determined by a micro-Kjeldahl procedure) to absorbance. From 0.03 to 0.16 mgm. of protein N per tube could be determined using this method.

TABLE 2

Liquefying and saccharifying power of dialyzed milk under various conditions

Lot	Heat treatment	Incubation conditions Temp.	pH [a]	Salts added	Conc. EDTA [b]	Liquefying power	Saccharifying power [c]
		(° C.)			*(molarity)*	*(μA/ml)*	*(mgm. maltose hydrate/ hr/liter)*
1. Whole	None	34	6.2	0.06M NaCl	0.0002	19.0	—
Herd	None	34	6.2	0.06M $CaCl_2$	0.0002	22.4	—
	None	34	7.3	0.06M $CaCl_2$	0.0002	26.7	—
2. Whole	None	48	6.4	0.06M NaCl	0.0002	34.0	1,700
Commercial	None	48	6.4	0.06M NaCl	0.0023	9.0	—
	None	48	6.4	0.06M NaCl	0.0042	3.0	—
	None	48	6.4	None	0.0002	1.6	—
3. Whole	None	48	6.4	0.06M NaCl	0.0002	39.0	2,500
Herd	None	48	6.4	0.06M NaCl	0.0042	4.1	270
	57.5° C. 30 min.	48	6.4	0.06M NaCl	0.0002	2.2	120
4. Skim	None	37.5	6.4	0.03M NaCl	None	—	1,380
Herd	None	37.5	6.4	None	0.0031	—	193
	53.5° C. 30 min.	37.5	6.4	None	0.0031	—	193
	68 ° C. 30 min.	37.5	6.4	None	0.0031	—	Negligible
	None	37.5	5.1	None	0.0031	—	132

[a] pH controlled by following buffers: pH 6.2 and pH 6.4—phosphate, pH 7.3—THMAM, pH 5.1—acetate. The concentration of buffer in reaction mixture was $\mu = 0.1$.

[b] Concentration of disodium ethylene diamine tetraacetate in incubation mixture.

[c] Measured by picric acid method.

EXPERIMENTAL PROCEDURE

Liquefying and saccharifying activities of dialyzed milks. Although the liquefying power of unmodified milk can be determined readily, direct measurement of its saccharifying power would necessitate a large correction for the lactose. Furthermore, bacterial growth might occur at the incubation times and temperatures used. Consequently, milks were dialyzed in the presence of toluene at 3° C. *vs.* 5×10^{-4} *M* disodium ethylenediamine tetraacetic acid (EDTA) until virtually free of lactose, and their liquefying and saccharifying powers measured. Results are shown (Tables 1 and 2).

Dialysis markedly increased the liquefying activity of two samples, but did not greatly affect that of eight others; no explanation is evident for this difference. Heat treatment at 57.5° C. for 30 min., or further addition of EDTA, suppressed liquefaction and saccharification in a like manner. Heat treatment very likely denatures the enzyme. Since all of the Ca^{++} probably was not removed by the dialysis, and since calcium and chloride are both necessary for the activity of the α-amylase *(13)*, the effect of the additional EDTA probably was dúe to sequestration of this ion. For some, but not all dialyzed milks, a small but measurable saccharification was observed in the presence of EDTA and absence of any activators (Sample 4, Table 2). This activity, which appears to represent a β-amylase, was carefully measured by using as controls both milk heated to 68° C. for 30 min. and a mixture of separately incubated milk and starch plus buffer. Samples were incubated for from 12 to 24 hr. under toluene, to measure this low activity.

TABLE 3

Distribution of alpha-amylase activity among whole milk, skimmilk, acid whey, lactoglobulin, and lactalbumin

Lot	Liquefying power[a] (*μA per ml. whole milk*)				
	Whole	Skim	Acid whey	Lacto-globulin[b]	Lactal-bumin[c]
1. Mixed milk, 4 cows	.....	9.9	.....	4.5	3.8
2. Mixed milk, commercial	19.0	19.7	.....	.....	.....
3. Mixed milk, commercial	13.5	14.1	9.1	6.1	Negligible
4. Mixed milk, commercial	17.4	19.8	16.2	6.2	Negligible
5. Mixed milk, commercial	15.6	16.7	9.5	10.4	Analysis unsatisfactory
6. Mixed milk, commercial	.....	15.0	8.9	9.4	1.0

[a] All determined in presence of 0.06 or 0.075 *M* $CaCl_2$, 34° C., pH 7.3–7.4, (THMAM buffer), 3.0% starch substrate.
[b] Precipitate obtained from whey protein at pH 6.2, 43% saturation with $(NH_4)_2SO_4$.
[c] Filtrate remaining after precipitation of lactoglobulin.

Distribution of alpha-amylase in milk protein fractions. Fractionation experiments in an attempt to concentrate the α-amylase were performed with small samples of mixed milk (Table 3). The α-amylase was concentrated in the skimmilk upon separation, and in the whey upon precipitation of the casein with acid, although some of it followed the casein. Furthermore, it came down to a large extent with the lactoglobulin fraction upon precipitating the latter at pH 6.2

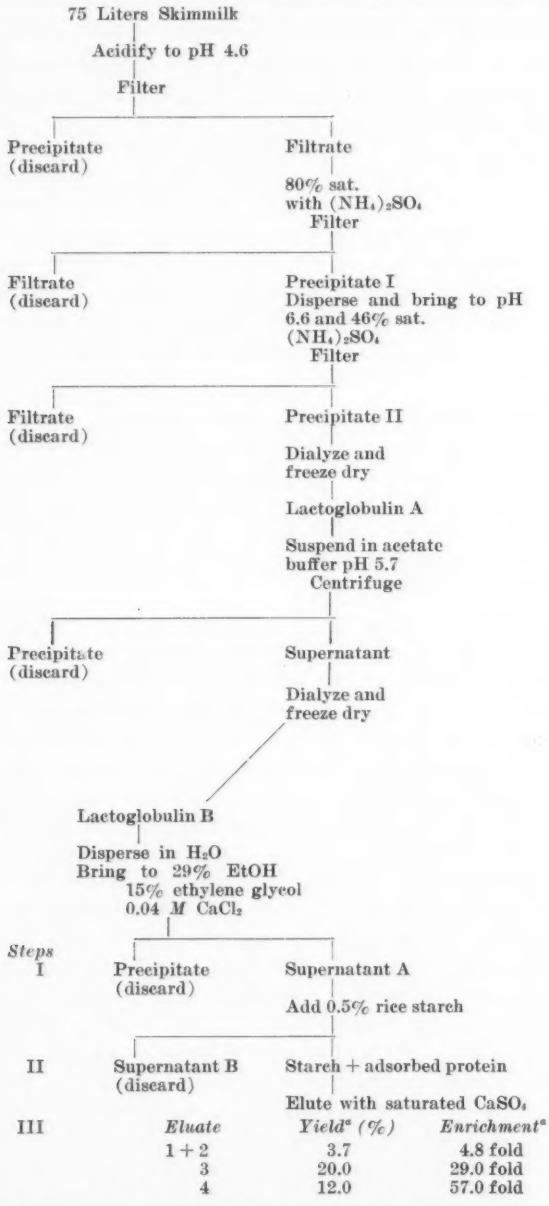
with 43% saturated ammonium sulfate, although in one case (Lot 4) considerable enzyme was lost in this fractionation. Thus, the lactoglobulin fraction was used as the starting material in attempts to concentrate and purify the enzyme.

Concentration of α-amylase from lactoglobulin. Concentration of α-amylase was based upon the work of Holmbergh *(4)*, in which the enzyme was dissolved in 30 or 40% ethanol, adsorbed upon rice starch at 0° C., and eluted from the starch by shaking with water. When this procedure was applied to lactoglobulin, only from 14 to 38% of the α-amylase could be dissolved in the ethanol solution. However, inclusion of 15% ethylene glycol (v/v) solubilized up to 86% of the α-amylase. Moreover, when rice starch was added to this mixture, up to 94% of the solubilized α-amylase was adsorbed on it, and when eluted from the starch with water, the α-amylase was concentrated up to 57-fold (12% yield based on activity of the lactoglobulin). With one sample, an enrichment of 240-fold over the activity of the lactoglobulin was obtained by two successive adsorptions and elutions.

Samples of the lactoglobulin fraction were isolated in large amounts and assayed for α-amylase. On the basis of preliminary kinetic studies of these samples, α-amylase activity was followed during fractionation by determining liquefaction at 48° C. on 3.20% potato starch paste at pH 6.2 with acetate buffer ($\mu = 0.01$) in the presence of 0.06 *M* $CaCl_2$. A lot of lactoglobulin containing a relatively high concentration of α-amylase and some apparent β-amylase activity was chosen for further fractionation.

The steps employed in the purification of α-amylase are summarized in Figure 1 and in the following paragraphs. Seventy-five liters of skimmilk from the University herd was acidified to pH 4.6 by slowly adding 4 liters of 1 *N* HCl with stirring. The next morning the casein was filtered off through washed cotton sacks and filter paper. To 58 liters of the whey, 71.1 lb. of $(NH_4)_2SO_4$ (80% saturation) were added. After being held overnight, the mixture was filtered and the precipitate, I, dispersed in water to give 16 liters of total volume. Ten liters of saturated $(NH_4)_2SO_4$ was added (46% saturation) and the pH adjusted to 6.6 with 1 *N* NaOH. The mixture was filtered and the precipitate, II, was held 4 wk. at 3° C. under toluene. It was then exhaustively dialyzed against both tap and distilled water until free of $SO_4^=$. The contents were frozen, pulverized, and freeze-dried in an apparatus described by Nickerson, Coulter, and Jenness *(9)*. Eighty-seven grams of the material thus obtained (designated Lactoglobulin A) was stored at 3° C. for a month. The preparation was suspended in acetate buffer ($\mu = 0.1$), pH 5.7, and allowed to stand for 2 hr., following which it was centrifuged to remove a small amount of a lipoprotein fraction. The supernatant, containing the active enzymes, was filtered through qualitative filter paper, exhaustively dialyzed under toluene against distilled water at 3° C., and again freeze-dried. Seventy grams of this purified lactoglobulin (designated Lactoglobulin B) were obtained. It had an α-amylase activity of 25.0 μA/mgm *N* and 1.2 mgm. maltose hydrate/hr/mgm *N* at 48° C. and pH 6.2. It was fractionated further by the following steps:

FIG. 1. Purification of Alpha-Amylase.



[a] Yield and enrichment calculated on basis of Lactoglobulin B.

Step I. About 16.0 g. of Lactoglobulin B were suspended in 610 ml. of 0.08 *M* $CaCl_2$ solution at 3° C., and 180 ml. of ethylene glycol at 3° C. was slowly added. The next morning, 420 ml. of 83% ethanol (v/v) precooled to 3° C. was added dropwise from a separatory funnel over a 15-20 min. period while stirring the mixture. After 4 hr., the mixture was centrifuged at high speed for 15 min. in a Servall centrifuge and the precipitate washed twice with 100 ml. of the solution containing 29% ethanol and 15% ethylene glycol. The supernatant (designated A) contained 86% of the total α-amylase (enrichment 1.1-fold) and 96% of the β-amylase (enrichment 1.2-fold).

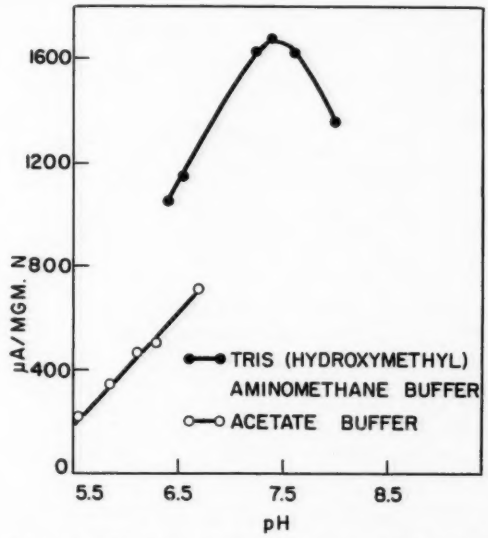


FIG. 2. Effect of pH on liquefying activity of purified alpha-amylase.

Starch concentration	= 3.20%
$CaCl_2$	= 0.075 *M*
Temperature	= 34° C.
Reaction time	= up to 30 min.

Step II. Approximately 6.0 g. of pulverized rice starch (Morningstar Nicol, Inc.) was added to 1,200 ml. of the Supernatant A; two days later the starch was centrifuged at 2,500 r.p.m. for from 20 to 30 min. The supernatant (designated B) contained 93% of the total β-amylase and 17% of the total α-amylase activity.

Step III. The starch, carrying 69% of the α-amylase, was eluted with four portions of saturated $CaSO_4$ solution. After each elution, the starch was centrifuged and the supernatant, containing the α-amylase, was dialyzed. The first elution was made by stirring the starch with 30 ml. of solution for about a minute. The second elution was made by stirring for one-half hour with 50 ml. of solu-

tion. The third and fourth elutions were made by stirring for periods of up to an hour with 75 ml. of solution. The yield of enzymatic activity is shown (Figure 1).

Properties of Concentrated Enzyme. A lot of purified α-amylase, prepared by the scheme outlined in Figure 1, was chosen for further study. It was enriched about 30-fold, compared to the lactoglobulin from which it was isolated, having a liquefying activity at 34.3° C. and pH 7.4 of 1,750 μA/mgm *N*, saccharifying activity of 149 mgm. maltose hydrate/hr/mgm *N*, and dextrinifying activity of 115 mgm. starch 30/min/mgm *N* (11.5 Smith and Roe units per mgm *N*).

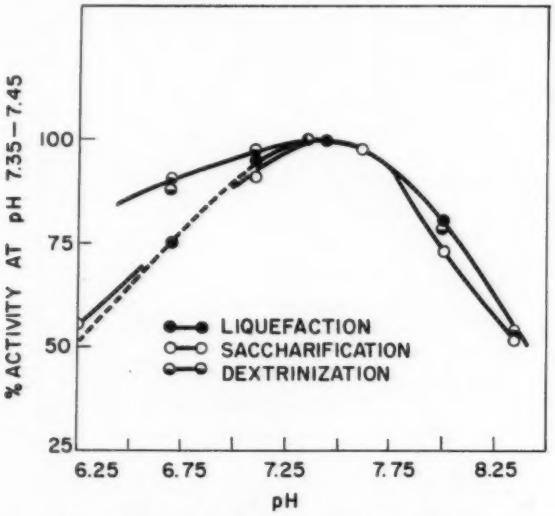


FIG. 3. Effect of pH on saccharifying, dextrinizing, and liquefying activities of purified α-amylase.

Starch concentrations = saccharification 1.12%, dextrinization 0.6%, liquefaction 3.20%
$CaCl_2$ = 0.075 *M*
Temperature = 34° C.
Reaction times = Saccharification to 65 min., dextrinization to 80 min., liquefaction to 30 min.
Buffer = Tris(hydroxymethyl)aminomethane (THMAM)

A preliminary check of the effect of temperature on the liquefying action of this lot was made at pH 6.2, acetate buffer, $\mu = 0.1$, 3.20% starch, 0.06 *M* $CaCl_2$, and 30 min. reaction time. This series indicated an optimum temperature of about 44° C. Hence, in further work, a temperature of 34° C. was adopted, in order to avoid complications due to inactivation of the enzyme.

Effect of pH. The effect of pH on the liquefying activity was determined at 34° C. over the range from 5.5 to 8.0, with acetate and THMAM buffers. The reaction mixtures had a buffer ionic strength of 0.1 and $CaCl_2$ concentration of 0.075 *M*. The data (shown in Figure 2) indicate optimum activity at pH 7.4.

The reason for the greater activity in THMAM buffer at pH 6-6.5, as compared to acetate at the same pH, is not known, although such effects are often noted in enzyme chemistry.

The effect of pH on the saccharifying and dextrinifying activities was also determined with THMAM buffers. The results (plotted in Figure 3) indicate that the optima for all three measures of activity coincide at pH 7.4.

Effect of temperature and energy of activation. The liquefying activity of the α-amylase preparation was determined at temperatures between 25 and 49° C. at pH 7.4. In all cases, the plots of $1/\eta_{sp}$ *vs.* time were linear up to 30 min. reaction time, which is indicative of a zero-order reaction. A plot of log of the reaction rate (actually, log μA, which is proportional to the log rate) *versus* the reciprocal of the absolute temperature, exhibits (Figure 4) a linear relationship

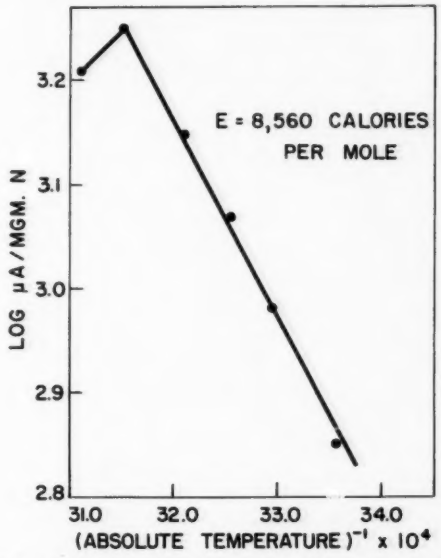


FIG. 4. Effect of incubation temperature on the rate of liquefaction of starch by purified α-amylase.

Starch concentration	= 3.2%
$CaCl_2$	= 0.06 *M*
pH	= 7.3—7.4 (THMAM buffer)
Reaction time	= up to 30 min.

up to 44° C., beyond which denaturation causes a reduction in activity. The energy of activation, calculated from this plot by the Arhennius equation, log rate = $-E/2.303\,RT + C$, is 8,560 calories per mole.

Effect of heat treatment. A lot of the purified enzyme and two lots of lactoglobulin were heated in solution in acetate buffer at pH 6.4, $\mu = 0.1$ for 30 min. at various temperatures. Likewise, a sample of skimmilk was given similar heat treatments. Liquefying activities as percentages of the activity of the respective

unheated controls are plotted against temperature (Figure 5). All four samples exhibited a sharp reduction in activity in the range 45-52° C., with half of the activity being destroyed by heating at about 50° C. for 30 min.

Effect of substrate concentration. The effect of starch concentrations ranging from 0.06 to 0.6% on the rate of saccharification was determined. A plot of these data by the method of Hofstee *(3)*, *V. vs. V/S*, yielded a straight line (shown in Figure 6). Linearity of such a plot indicates that only one enzyme is present. In the Hofstee plot, the slope is equal to $-K_m$, the Michaelis constant, and the intercept on the ordinate is the maximum velocity. For this α-amylase preparation, K_m is 2.05×10^{-3} g. starch/ml on a 14.7% moisture basis, or 1.75×10^{-3} g. dry starch/ml. The maximum velocity is 140 mgm. maltose hydrate/hr/mgm *N*.

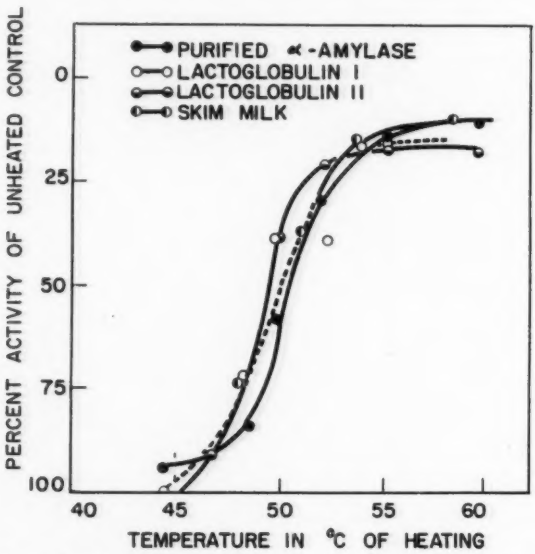


FIG. 5. Effect of 30-min. heat treatments upon the starch liquefying activity of purified α-amylase, lactoglobulin, and skimmilk.

Starch concentration	= 3.2%
$CaCl_2$	= 0.06 *M*
pH	= 7.10 (THMAM)
Temperature	= 34° C.
Reaction time	= up to 30 min.

The effect of substrate level on the rate of liquefaction by this amylase preparation was also studied. The activity appeared to increase at starch concentrations below 2%, but the viscosity method is not very sensitive at lower than 1% starch. Thus, no measurement of K_m could be made by this method.

Effect of activators on the liquefying and saccharifying activity. The effect of $CaCl_2$, calcium acetate, iodine, NaCl, $MgCl_2$, and cysteine HCl upon the liquefying and/or saccharifying activity of α-amylase was determined. The

results (shown in Table 4) indicated that optimal activation is attained for liquefaction by 0.075 *M* $CaCl_2$; NaCl and $MgCl_2$ of comparable ionic strength activate the enzyme to only 10% of the level attained with $CaCl_2$. Calcium acetate is without activation effect. Data also indicate that NaCl, or calcium acetate, activates no saccharification, whereas $CaCl_2$ does. However, the apparent total absence of activity may be a reflection of the dinitrosalicylic acid method used, in which small levels of sugar (0.18 mgm. maltose hydrate) are not detected. The addition of iodine to the starch substrate decreased the effects of both the liquefying and saccharifying activities by approximately the same amount, respectively, 33 and 31%. Cysteine did not elicit additional saccharifying activity.

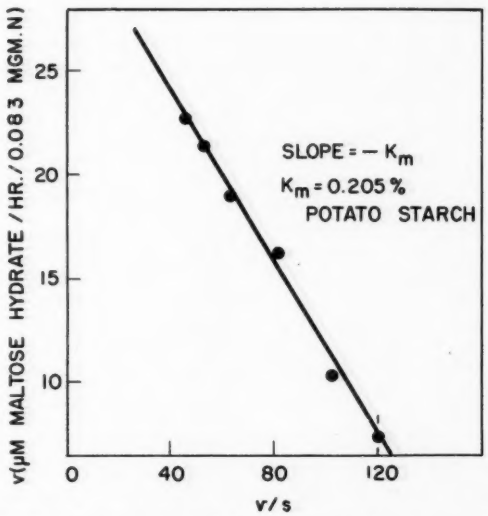


FIG. 6. Plot of velocity *vs.* velocity/substrate concentration for saccharifying activity of purified α-amylase.

Starch concentrations	= 0.06 − 0.60%
$CaCl_2$	= 0.5 *M*
pH	= 7.4 (THMAM)
Temperature	= 34° C.
Reaction time	= up to 120 min.

All velocities extrapolated to zero-time.

DISCUSSION

This work confirms that of Chrzaszcz and Goralowna *(1)*, Sato *(12)*, Schloemer *(13)*, and others in showing that cows' milk contains an α-amylase. It supports the findings of Richardson and Hankinson *(11)* by indicating that at least some lots of cows' milk contain weak β-amylase activity.

The present findings also corroborate those of Schloemer *(13)* in showing that both Ca^{++} and Cl^- ions are necessary for the activation of this α-amylase, and that it largely follows the whey protein fraction of milk. Furthermore, the

reports of previous workers *(11, 13)*, that this is a very heat-labile enzyme, have been confirmed. This heat lability was demonstrated both in milk and in concentrated preparations.

The purified α-amylase prepared was believed to be free from β-amylase, because *(1)* no saccharification was detected in the absence of both calcium and chloride, *(2)* a plot of velocity *vs.* velocity/concentration of substrate, according to the method of Hofstee, yielded a straight line indicative of one component, *(3)* in the fractionation of lactoglobulin negligible β-amylase was adsorbed upon rice starch, *(4)* negligible additional saccharification was observed when cysteine hydrochloride, a sulfhydryl activator, was included in addition to the $CaCl_2$, and *(5)* the decrease in liquefaction caused by the iodine was paralleled by the decrease of saccharification.

TABLE 4

Effect of different activators and inhibitors on the liquefying and saccharifying activity of purified alpha-amylase

Adjunct[a]	pH[b]	Ionic strngth[c]	Activity[d]	
			(μA/mgm N)	*(mgm. maltose hydrate/hr/ mgm N)*
1. None	7.65	0.035	None	None
2. NaCl—0.10 *M*	7.65	0.135	171	None
3. $MgCl_2$—0.075 *M*	7.4	0.260	196	.....
4. $CaCl_2$—0.060 *M*	7.4	0.215	1690	.....
5. $CaCl_2$—0.075 *M*	7.4	0.260	1750	149
6. $CaCl_2$—0.090 *M*	7.4	0.305	1740	.....
7. Ca acetate—0.075 *M*	7.4	0.260	None	None
8. Cysteine HCl—0.0008 *M* +$CaCl_2$—0.075 *M*	7.4	0.261	.....	152
9. Iodine—KI—.00024 *M* I_2+$CaCl_2$—0.075 *M*	7.4	0.260	1170	103

[a] Concentration in reaction mixture.

[b] The effect of Ca and Mg on the pH of the buffer was not compensated.

[c] Including THMAM buffer of ionic strength $\mu = 0.035$. In Experiments 1 and 7 the THMAM buffer was used as the acetate, in all other experiments as the chloride.

[d] All incubations at 34° C. Dinitrosalicylic acid method used for saccharification; 3.20% starch and 30 min. incubation used for liquefaction, 1.50% starch and 150 min. incubation used for saccharification.

Alpha-amylase from cows' milk differs from those from other sources by its dependence upon both the chloride and calcium ions for maximal activity. Those from plant sources require calcium; whereas, in general, those of animal or microbial source require chloride ions. The pH optimum of 7.4 found for the milk enzyme resembles that of amylase from human blood (6.8–7.6).

Large amounts of milk would have to be worked up if further purification of the α-amylase were to be attempted. Thus, one liter of milk containing 18,000 μA units yields, assuming a 50% loss, about 1.25 g. of lactoglobulin, with a specific activity of about 45 μA/mgm *N*. This lactoglobulin yields, in turn, with about 60% loss (see Figure 1), a concentrate enriched about 40 times with a specific activity of about 1800 μA/mgm *N*. Even this concentrate has a specific activity of only 1/200th to 1/500th that of crystalline α-amylases from other sources.

Although glycerol is known to be a good solubilizing agent for enzymes, the authors are not aware of the previous use of ethylene glycol for this purpose. It proved very effective in controlling the solubility of α-amylase in ethanol solution. Moreover, by this addition, the concentration of the ethanol could be kept high enough to cause adsorption of the enzyme upon added starch.

ACKNOWLEDGMENT

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DETERMINATION OF UNOXIDIZED TOCOPHEROLS IN MILK FAT[1,2]

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SUMMARY

This procedure was evolved in order to follow tocopherol degradation in oxidizing milk fat. It combines saponification, in the presence of pyrogallol, to destroy tocoquinones and essentially all fat peroxides, chromatography on $SnCl_2$ + HCl-treated Floridin (Kjolhede) to remove carotenoids, possible residual peroxides, steroids, etc., and color development in benzene-ethanol with ferric chloride and bipyridyl in acetic acid solution (Devlin and Mattill). Pyrogallol was effective in preventing tocopherol destruction during saponification and was superior to saponification under nitrogen. Complete saponification was effected by using 17 ml. of 3.5 *N* KOH per 10 g. fat. If saponification was eliminated, the treated Floridin column reduced tocopheryl-*p*-quinones but not tocopheryl-*o*-quinones. The former were similarly reduced by the Quaife and Biehler hydrogenation procedure. These reduced compounds are measured as tocopherols.

A method was needed for the determination of unoxidized tocopherols present in milk fat, in order that their destruction during oxidation of milk fat samples in storage could be followed. It was likewise considered that the amount of destruction of tocopherols might be employed as a criterion of degree of oxidation of the fat.

A literature survey indicated that many methods had been employed for tocopherol determination in fatty materials. The most common was that of Emmerie and Engel *(5)*, together with its many modifications. The procedure that will reported combines certain aspects of those of Devlin and Mattill *(3)*, Moore and Tosic *(8)*, and Kjolhede's modification *(7)* of Emmerie and Engel's method *(5)*.

EXPERIMENTAL PROCEDURE

In the search for simple methods, those of Quaife and Biehler *(10)*, Parker and McFarlane *(9)*, and Kjolhede (with whole fats) *(7)* were tried. The first was unsatisfactory because of the reduction of tocoquinones present in the oxidized fat; the second caused troublesome emulsions with some fats, and the third yielded high values with oxidized fats, presumably because of reduction of tocoquinones on the column.

The reason for selecting the combination indicated above resulted from the fact that Devlin and Mattill's *(3)* use of benzene-acetic acid in place of ethanol caused slower color development, improved lipid solubility, and eliminated

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troublesome light-catalyzed oxidation of ethanol by ferric iron. Interfering tocoquinones and peroxides were removed by saponification of the sample in the presence of pyrogallol *(8)*. Interfering carotenoids, vitamin A, steroids, and possibly other materials were removed from benzene solutions of nonsaponifiables by use of specially treated Floridin *(7)*.

Tocopherol determinaton. Saponification and extraction of sample. Mix 10 g. of fluid fat and 5 ml. pyrogallol solution (5 g. to 100 ml. reagent absolute methanol) in ST. 150-ml. actinic extraction flask, add 20 ml. 3.5 *N* absolute methanolic KOH, reflux on an 80° C. water-bath for 10 min. after the solvent begins to drip from the condenser tip. Remove heat, rinse condenser into saponification mixture with 5 ml. absolute methanol, remove flask, add 40 ml. distilled H_2O, mix, add 15 ml. absolute methanol, stopper, and cool in a room-temperature water-bath. Transfer to a 250 ml., g. s., amber separatory funnel, rinse saponification flask with small portions (aggregate 60 ml.) distilled water, and small portions (aggregate 80 ml.) of peroxide-free ether.[5] Shake vigorously ten times, remove ether layer, and extract the aqueous layer twice more with 50 ml. peroxide-free ether. Combine extracts and wash successively, with 50 ml. 1% aqueous KOH, 50 ml. 2% Calgon, and two 50-ml. portions of distilled water. Dry over Na_2SO_4. Remove ether from dried solution under vacuum, introduce 2 ml. benzene[6] through the stopcock of the evaporation apparatus *(14)*, release vacuum, and make residue to volume in a 25-ml. volumetric flask.

Tocopherol analysis. Prepare Floridin columns,[7] chromatograph benzene solutions of nonsaponifiables (equivalent to 4 g. fat), and evaporate combined percolate and washings by the Kjolhede method *(7)*, except that in eluting the columns use five, 5-ml. portions of benzene.

To develop the ferrous-bipyridyl color, transfer the residue from the last evaporation of the percolate and washings, quantitatively to a 25-ml., actinic red, g. s., graduated cylinder with benzene, make to the 10-ml. mark with benzene, add 2 ml. of Devlin and Mattill reagent *(3)*, make to 25 ml. with absolute ethanol,[8] invert three times, pour into a cuvette, and read the percentage transmittance (Coleman Model 11 Spectrophotometer) at 520 mμ, 5 min. after the last of the reagent is delivered from the pipette, against a blank (prepared

[5] Peroxides were determined by a modified Chapman and McFarlane method *(1)*: Dissolve 0.2 g. NH_4SCN in 2 ml. distilled H_2O and make to 50-ml. volume with purified acetone. Invert flask into a folded Whatman No. 40 filter containing 0.1 g. $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$. Catch filtrate in a second 50-ml. amber volumetric flask. Place 5 ml. reagent and 5 ml. purified acetone in a color-matching tube as the blank; use 5 ml. reagent and 5 ml. ether in a second tube as the peroxide test.

[6] Reagent, thiophene-free benzene, was refluxed over 20 g. KOH and 5 g. aluminum per liter and distilled; the first 50 to 100 ml. of a 4-liter batch were discarded.

[7] The Floridin XXS used was furnished by The Floridin Co., Warren, Pa. The tubes employed were 12-cm. pieces of 10-mm. tubing, joined at one end to 5-cm. pieces of 5-mm. tubing and at the other to 10-cm. pieces of 16-mm. tubing.

[8] Refluxed over 20 g. KOH pellets and 2 g. $KMnO_4$, per liter for 1 hr.; discard the first and last 300 ml. of 4-liter batch.

without sample) set at 100% transmittance. The tocopherol quantity can be calculated by use of a standard regression equation.[9]

Milk fat was prepared from unsalted butter. The butter was churned from 40% cream (obtained from the Iowa State College Market Milk Department) in quart Mason jars with glass lids and rubber gaskets, in a shaker with reciprocating action in the direction of the long axis of the jar. After sharp-freezing for 24 hr., the butter was melted (40-45° C.), centrifuged to separate the fat from the aqueous phase, the fat filtered through Whatman No. 12 folded filters, in heated funnels (fat temperature *ca.* 45° C.), and collected and stored in 4-oz. aluminum, screw-capped, glass sample bottles. Fats were stored at approximately −25° C. until used. The samples which showed peroxide values (Table 1) had been in storage for from 12 to 15 mo.

TABLE 1

Comparison of the saponification procedure employed in the vitamin A method with that employing 3.5 N methanolic KOH

Sample aliquot	Saponification procedure	Pyrogallol added	Tocopherol value (γ/g fat) Kjolhede	Peroxide value (m.e/kg) Kjolhede	Peroxide value (m.e/kg) Untreated[a]
1	Vitamin A[b]	+	46.4–46.0	0.00	0.012
2	Vitamin A[b]	+	45.8–46.7	0.00	0.007
3	Vitamin A[b]	0	30.6–31.2	0.00	0.012
4	Vitamin A[b]	0	31.0–32.2	0.00	0.009
5	Not saponified	0	55.4–56.6	0.00	3.19
6	3.5 *N* KOH	+	46.7–45.8	0.00	0.010
7	3.5 *N* KOH	+	44.9–46.0	0.00	0.015
8	3.5 *N* KOH	0	32.4–32.8	.....	.....
9	3.5 *N* KOH	0	29.6–30.2	.....	.....
10	Not saponified	0	57.3–55.8	0.00	3.02

[a] Nonsaponifiables or whole fat not passed through absorbent column.
[b] Methanolic KOH, 4 *N*.

RESULTS AND DISCUSSION

Preliminary use of the Emmerie *(4)* saponification technique indicated that it yielded various degrees of saponification (indicated by volumes of nonsaponifiable residue) and erratic results. Chipault *et al. (2)* indicate that 17 ml. of 3.5 *N* methanolic KOH were found to leave but a few drops of nonsaponifiables from 10 mg. of milk fat. A comparison of 3.5 *N* and a 4.0 *N* (U. S. Technical Committee on Vitamin A Researches) methanolic KOH indicates that results with both alkalies are essentially the same (Table 1). These data likewise confirm the protective action of pyrogallol, and show that the saponification procedure destroys most of the interfering peroxides in the fat.

Difficulties were encountered in obtaining adequate recoveries of D-α-tocopherol, when added to milk fat as a solution in benzene. When added directly to milk fat as the solvent, satisfactory recoveries (from 90 to 94%) were obtained.

[9] Standards were prepared from pure D-α-tocopherol (Distillation Products, Inc.). Benzene solutions containing 2 to 400 γ were treated for color development as described in "Tocopherol Analysis." Regression equations were calculated from the data.

TABLE 2

Comparison of the effects of nitrogen and pyrogallol on tocopherol contents of milk fat

Sample aliquot	Saponification treatment		Tocopherol (γ/g fat)	
	Pyrogallol	Nitrogen	Chromatographed[a]	Not chromatographed[b]
1	+	−	39.4	41.1
2	+	+	42.5	40.7
3	−	+	32.2	.....
4	−	−	25.7	22.9
5	+	−	44.0	41.5
6	+	+	44.0	39.5
7	−	+	24.4	22.4
8	−	−	26.6	25.9

[a] Nonsaponifiables passed through Kjolhede-Floridin column.
[b] Nonsaponifiables run without passing through column. Carotene corrections made by method of Quaife *et al. (11)*. These samples were fresh fats in which no oxidation had occurred.

TABLE 3

Degree of interference of p-quinone in the tocopherol determination

Treatment	γ p-quinone added per g. of fat	γ tocopherol per g. of fat	Difference (sample minus control)	% conversion of p-quinone added	% error (basis, 65.4 mcg. per g.)
Kjolhede	71.2	103.2	37.8	53.0	57.9
Kjolhede	35.6	93.6	28.2	79.3	43.2
Kjolhede	17.8	74.0	8.6	48.4	13.2
Kjolhede[b]	0.0	65.4	.....	.....	.....

[a] Whole fat passed through Kjolhede-Floridin columns.
[b] Control.

TABLE 4

Effect of saponification on the degree of interference of added p-quinone in the tocopherol determination

Sample aliquot	5 ml. of 5% pyrogallol added	γ p-quinone added per g.	Tocopherol (γ/g fat)	
			Kjolhede	Average
1 (Saponified)	+	40	54.0	53.2
2 (Saponified)	+	40	51.5	
3 (Saponified)	+	0	49.2	47.9
4 (Saponified)	+	0	46.6	
5 (Saponified)	−	40	34.0	34.0
6 (Saponified)	−	40	.....	
7 (Saponified)	−	0	33.0	34.5
8 (Saponified)	−	0	36.0	
9 (Whole fat)	−	0	51.3	
10 (Whole fat)	−	66	68.2	

Initially, it was the intent to minimize oxidative effects by saponifying samples in a nitrogen atmosphere. Erratic results were obtained. The use of pyrogallol *(8)* eliminated this variability (Table 2). These data, obtained with fresh fats, indicate that a nitrogen atmosphere is not necessary when pyrogallol is employed. A comparison of the values obtained for saponified and chromatographed fat aliquots, and those obtained for saponified aliquots which were not chromatographed but were corrected for carotene interference *(11)*, indicated that chromatography of the nonsaponifiables from fresh fats corrects only for carotene interference.

TABLE 5

Assay[a] of hydrogenated solutions of p-quinones

γ of *p*-quinone	Standing time *(min.)* after hydrogenation	γ found as tocopherol	% recovery of added *p*-quinone
333	5	157.5	47.3
333	10	134.4	40.4
333	15	161.3	48.4
333	60	68.9	20.7
333	120	44.5	13.4

[a] Quaife and Biehler method *(10)*.

Kjolhede-treated Floridin removes peroxides[10] from fat or nonsaponifiable fractions (Table 1). However, when whole oxidized fats are passed through these columns, tocopherol values are obtained that are higher (Aliquots 5 and 10) than when these fats are saponified with pyrogallol and then chromatographed. It was considered that the elevation of the values might result from reduction of *p*- or *o*-quinones[11] resulting from tocopherol oxidation. Consequently, natural D-α-tocopherol was oxidized with ceric sulfate *(12)* to produce the *p*-quinone, and by nitric acid *(13)* to produce the *o*-quinone. The *p*-quinone itself did not react with the Devlin and Mattill reagent, but when added to milk fat in quantities which might be found in oxidizing milk fat, assays of whole fat passed through Floridin columns increased by amounts equivalent to from 13.2 to 57.9% of the tocopherols naturally present (Table 3). The *o*-quinone, however, caused no appreciable difference in whole fat assays. Saponification of samples, to which the *p*-quinone was added, eliminated appreciable interference by the *p*-quinones (Table 4).

The data of Table 5 confirm the statement that the *p*-quinones are reduced to a considerable degree in the Quaife and Biehler *(10)* hydrogenation and are measured as tocopherols. They likewise indicate that the hydroquinones presumably formed are slowly oxidized.

[10] Peroxides were determined by the Hills and Thiel method *(6)*, except that the reaction mixture was heated at 60° C. for 3 min. and, in order to minimize weighing errors and speed the determination, 1 g. fat was dissolved in benzene-methanol and aliquots were pipetted. In addition, the developed color was read in a Coleman 11 Spectrophotometer and peroxide values were calculated from standard regression equations.

[11] Tocopheryl-*o*-quinone and tocopheryl-*p*-quinone.

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FORMATION OF BACTERIAL INHIBITORY ZONES IN WHEY AGAR BY RAW MILK

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SUMMARY

This investigation is concerned with the nature of bacterial inhibitory zones of milk, their incidence, and a technique for measuring the antibacterial property of raw milk. The disc assay test for antibiotics in milk was adaptable for the detection of natural inhibitors in raw milk. By substituting lyophilized milk tablets for the saturated paper discs, the sensitivity of the test was increased. Generally, raw milk produced two clearly defined zones, labelled primary and secondary, respectively, against *Bacillus subtilis*. A primary zone of inhibition first appeared after 12 hr. at 72° F. The secondary zone only appeared after the plates were incubated, refrigerated, and then reincubated. This secondary phenomenon caused disappearance of the bacterial colonies like that associated with lysis. It appears that the presence of the natural inhibitor in milk is independent of the breed of the cow. The initial zone size varied seasonally, and tended to decrease in diameter or disappear during the winter months, whereas secondary zones were less affected seasonally.

Freshly drawn cow's milk often exhibits a germicidal action. As early as 1901, Hunziker *(4)* showed that high-heat treatment of milk destroyed the bactericidal effect, and Chambers (1), in 1920, indicated that its specificity was dependent not only on the individual cow but also on the bacterial species. A literature review covering the period prior to 1927 was presented by Jones *(5)*. In general, the extent of the germicidal effect was measured by direct counting of bacteria or by lactic acid production.

During disc assay studies for antibiotics in milk *(6)*, small zones were noted with antibiotic-free samples. This zoning apparently was associated with some natural antibacterial property of the raw milk. The ability to observe this zoning phenomenon with disc assays stimulated a more complete investigation into the general subject, especially when it was noted *(8)* that the sensitivity of measurements could be significantly increased through concentrating the milk. This paper is concerned with the incidence and nature of the zoning phenomenon in raw milk.

METHODS

Milk samples. Raw milk samples, collected fresh from individual cows of the Cornell University dairy herd, were cooled to 40° F. and tested within 24 hr.

Test organisms. Spore suspensions of *Bacillus subtilis* [1] were of the type recommended for the disc assay procedure. When not obtained commercially,

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[1] Commercial preparations obtained from Difco Laboratories, Detroit, Michigan. Other strains of *B. subtilis* used were No. 8 and No. 9524.

spores were prepared by the method of Foster and Woodruff *(3)*, using a modification suggested by Silverman *(7)*.

Freeze-drying. A simple apparatus (shown in Figure 1) proved to be an efficient freeze-dryer. A temperature differential was maintained in the desiccator by placing its lower portion in the port of an ice cream cabinet at —18° C.

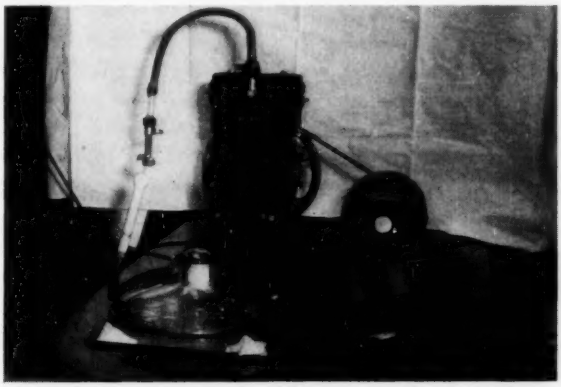


FIG. 1. Apparatus for freeze-drying milk samples.

The upper area was exposed to the atmosphere. The evaporated water condensed and formed a layer of ice on the bottom of the desiccator. This eliminated the need for a condenser between the vacuum tube and desiccator, but a calcium chloride tube was placed in this position as a precautionary measure.

Technique for detecting zones. Milk samples were concentrated to a fine white powder by freeze-drying, usually in less than 12 hr. Samples of this powder were compressed to a 13-mm. diameter circular tablet, weighing 0.1 g., in a Stokes [2] pill-maker. (Another simple way to form tablets is to place the desired weight of powder in a Pyrex-glass powder funnel and compress the powder into a tablet by pounding with a glass rod.)

The milk tablets were placed on the surface of seeded whey agar with a sterile spatula and assayed in a manner similar to that described for the standard paper disc assay method, without inverting the test plates. Resulting zone diameters, illuminated and magnified in a Quebec colony counter, were measured with calipers to the nearest 0.5 mm.

For comparative purposes, the standard disc assay method *(8)*, using 13-mm. paper discs, served as a control.

Lower incubation temperature for a longer period (20° C. for 12 hr.) was found to give slightly larger zones than at 37° C. for 6 hr., recommended in the standard disc assay test, and was more convenient for the analyst. The lower temperature was used throughout this study, but to detect antibiotics added to milk, the higher incubation temperature always is advocated.

[2] Stokes Machine Co., Philadelphia, Pennsylvania.

TABLE 1

Primary inhibitory zone displayed seasonally by raw milks [a]

Cow No.	Zone size																			
	1953		1954											1955						
	7/16 Disc	7/21 Disc	2/12 Disc	2/17 Disc	2/17 Tab.	4/22 Disc	5/6 Disc	5/6 Tab.	8/5 Disc	8/5 Tab.	9/30 Disc	12/28 Disc	12/28 Tab.	1/31 Disc	1/31 Tab.	2/12 Disc	2/12 Tab.	5/8 Disc	6/25 Disc	6/25 Tab.	7/18 Disc
	Diameter of zone minus diameter of disc or tablet *(mm.)*																			
1	2	2	**	**	**	0	1	2	1	5	3	<1	6	**	**	**	**	0	0	<1	0
2	4	2	**	**	**	0	1	4	2	5	3	*	*	0	6	0	4	0	0	<1	0
3	2	2	0	*	*	0	1	4	2	3	3	<1	<1	0	0	**	**	2	0	<1	0
4	5	*	0	0	0	**	*	6	3	4	3	<1	6	0	0	0	4	<1	0	5	0
5	*	*	0	0	*	1	1	*	4	*	3	<1	0	*	*	*	*	*	0	6	<1
6	2	2	**	**	*	0	<1	*	3	7	4	<1	6	**	**	**	**	*	0	<1	*
7	*	*	*	*	*	*	*	5	*	*	4	<1	0	0	0	0	0	**	*	*	*
8	*	*	*	*	*	*	*	*	*	*	4	<1	0	0	0	0	4	<1	0	6	1
9	*	*	*	*	*	*	*	*	*	*	3	<1	0	0	0	<1	0	2	0	8	2
10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	3	0	6	0
11	*	*	*	*	*	*	*	*	*	*	*	*	*	4[m]	6[m]	0	4	4	*	6	*
12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	4	0	*	3
13	*	*	*	*	*	*	*	*	*	*	*	*	*	0	0	0	0	*	0	8	*

Legend: 0—no zone; *—not tested; **—animal was dry; m—mastitic milk (Cow No. 11).

Disc—Test was made using 13-mm. diameter paper disc.

Tablet—Test was made using 13-mm. diameter dried raw milk tablet.

Values are averages of duplicate samples.

Cows 1, 6, and 11 are Holsteins; 7, 9, and 13 are Jerseys; and 10 and 12 are Brown Swiss.

[a] Against *Bacillus subtilis* bacteria grown on whey agar.

RESULTS

Effectiveness of technique. Replacement of paper discs with tablets of lyophilized milk showed, with many milks, natural zones where none was evident before. Also, zones previously observed in some raw milks were reproduced with significant increases in area, averaging about tenfold. The sensitivity of detecting bacterial inhibition by raw milks thus was materially improved (Tables 1 and 2).

Character of zoning. The phenomena of natural zoning of milks were more complex than previously had been anticipated. This became apparent inadvertently, when some finished plates displaying zones of inhibition were stored at low temperatures and later reincubated. Another type of zoning, distinct from the first, appeared on these plates.

With milks having the ability to inhibit bacteria, incubation at 20° C. for 12 hr. produced a zone of slight haziness averaging 15 mm. in diameter. This is called the primary zone (Table 1).

TABLE 2

Secondary inhibitory zone displayed seasonally by raw milks[a]

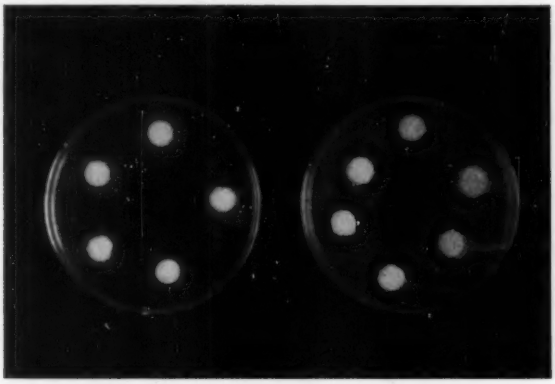
	Zone size										
	1954		1955								
Cow No.	12/28		1/21		2/12		4/24	5/25	6/25		7/18
	Disc	Tablet	Disc	Tablet	Disc	Tablet	Disc	Disc	Disc	Tablet	Disc
	Diameter of zone minus diameter of disc or tablet *(mm.)*										
1	4	14	**	**	**	**	7	0	0	14	0
2	*	*	4	15	3	10	**	0	6	15	5
3	4	15	3	14	**	**	**	0	0	11	0
4	5	15	2	9	0	13	**	0	0	9	0
5	4	19	*	*	*	*	*	1	3	0	*
6	2	15	**	**	**	**	8	0	0	15	2
7	4	17	0	12	0	10	*	**	*	*	*
8	4	19	0	11	0	11	*	2	0	0	0
9	4	19	<1	13	0	12	*	2	0	0	5
10	*	*	*	*	*	*	6	0	0	0	1
11	*	*	8[m]	18[m]	3	15	5	0	*	*	*
12	*	*	*	*	*	*	6	0	0	9	*
13	*	*	0	13	5	11	*	0	4	8	0

Legend: 0—no zone; *—not tested; **—animal was dry; m—mastitic milk.
Disc—Test was made using 13-mm. diameter paper disc.
Tablet—Test was made using 13-mm. diameter dried raw milk tablet.
Values are averages of duplicate samples.
Cows are same as in Table 1, except that No. 5 is a Jersey, instead of a Holstein.
[a] Against *B. subtilis* bacteria grown on whey agar.

Storage of this incubated plate at 5° C. for 24 hr., followed by reincubation at 20° C. for 12 hr., often resulted in another, more vivid, type of zoning (Figure 2). Formation, usually adjacent to the first zone, was apparent within 4 hr. after reincubation. The new circular zone began in an area of live bacterial colonies and worked its way back toward the milk tablet. In 12 hr. it had the ability to pass through the agar to this point, clearing all the haze apparent

in the primary zone. At times, however, it would reach only the periphery of the primary zone, leaving a ghost halo of living bacterial colonies separating two distinct zones, one slightly hazy, the other clear and transparent. This zone, averaging 20-mm. diameter through the center of the milk tablet, is hereinafter referred to as the secondary zone (Table 2). The secondary zone occurred also in milks not displaying the initial zoning.

The primary zone displayed characteristics normally associated with bacterial inhibition, but the secondary zone appeared to be associated with lysis of the test bacteria. Sporulation of bacteria may also account for the disappearance of colonies. However, very few spores were found, microscopically,



A_1 A

FIG. 2. An example of the secondary-zoning phenomenon encountered in present study. A. Concentrated raw milk tablets on whey agar, using *B. subtilis*. Note clear secondary zones. A_1. Concentrated heated milk tablets on whey agar, using *B. subtilis*. Note in A_1 the absence of primary and secondary zones as a result of heat treatment.

in secondary-zone agar areas stained with malachite green, thus strengthening the concept that lysis is responsible for the secondary zone.

Incidence and size of zoning. Milk from individual cows and from cows of different breeds of the Cornell University herd produced pronounced variations in primary-zone size (Tables 1 and 2). The largest primary zoning, 53 mm. in diameter, resulted from a sample of untreated mastitic milk, whereas winter milk from normal cows gave the smallest zones and lowest incidence. Secondary zoning was subject to much less seasonal variation.

In the winter of 1953, starting in December, no primary zones were obtained from mixed milk of the Cornell herd; none was found until late April of the next year. During the winter of 1954, the cows of the Cornell herd did not exhibit the primary zoning between December and April. When they were allowed outdoors, only in the barnyard, primary zoning was evident. They were not put on pasture until later in the season.

Secondary zoning exhibited less variation between milks of individual cows that did primary zoning. This also applied with milks obtained in different

seasons of the year. The ability of milk to create conditions resulting in primary and secondary zoning was not related to breed of cattle, for all breeds tested showed similar variations.

When pronounced natural inhibitory zoning is encountered in milk, the zone diameter may not express this concentration linearly, because the diffusion rate in agar becomes the limiting factor. Dilution of the raw milks, containing high concentrations before lyophilizing and assaying, restores true linearity.

TABLE 3

Dilution of raw milk with sterile distilled water and the resulting primary inhibitory zones[a]

Raw milk	Water	Raw milks tested		
		A	B	C
(ml.)		Diameter of zone minus diameter of disc *(mm.)*		
10	0	2	4	4
9	1	2	3	3
8	2	0	2	*
7	3	0	2	2
6	4	0	2	1
5	5	0	1	0
4	6	0	0	0
3	7	0	0	0
2	8	0	0	0
1	9	0	0	0
0	10	0	0	0

Values are averages of duplicate samples.

* Not tested.

[a] Against *B. subtilis* bacteria grown on whey agar.

Some milks (Table 3) show considerably more titer of natural inhibitory substances than do others. For example, at the point where the inhibitory effect was at a minimum, one milk required a dilution of nine parts of water to one part of milk; another 1:1, while a third sample was diluted 1.5:1.

DISCUSSION

In the present study, it was observed that some raw milks had the ability to produce two clearly defined types of zoning phenomena, analogous to those of staphylococcal hot-cold lysis, against sensitive bacteria. Only recently, Chevalier *et al. (2)* noted that inhibitory strains of streptococci grown as isolates in agar seeded with sensitive lactobacilli produced a similar type of zoning around each of their separate colonies. Here, then, is an example of raw milk and inhibitory streptococci which display in agar a similar behavior pattern against sensitive bacteria.

Agents such as lysozyme can lyse *B. subtilis* in a manner similar to that displayed by raw milk in the secondary zoning, but for reasons which are elaborated on in detail in a following paper, lysozyme apparently is not the causative agent with respect to milk.

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CHANGES IN THE BACTERIOPHAGE SENSITIVITY OF LACTIC STREPTOCOCCI

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SUMMARY

Twenty-three fast acid-producing pure cultures of lactic streptococci, propagated daily at 22° C. in skimmilk fortified with 0.75% nonfat milk solids, were tested periodically in litmus milk for changes in sensitivity to purified bacteriophage filtrates. The following changes occurred in the 10-mo. test period: *(1)* Six cultures became resistant to their homologous bacteriophages and sensitive to one bacteriophage. *(2)* Three resistant cultures became sensitive to one bacteriophage. *(3)* Six cultures became mixtures and required two bacteriophages to stop acid production. *(4)* Three cultures became resistant to their homologous bacteriophages and then were resistant to a mixture of those available. In all cases studied, except one, the bacteriophages were adsorbed only by sensitive cultures. The adsorption behavior of the bacteriophages, the types of change observed, and the methods used to prevent changes indicate that changes in sensitivity resulted from displacement of sensitive bacterial populations by resistant populations that arose from rare mutant bacteria. The rare mutants had a selective advantage and (in all cases except one) altered bacteriophage-adsorption characteristics (assumed to reflect changes in surface structure).

Luria and Delbruck *(8)* have shown that pure cultures usually contain resistant mutants that give rise to resistant bacterial populations after the cultures are attacked by bacteriophages. Resistant mutants arise independently of bacteriophage action and may differ from sensitive cells by only a simple, gene-controlled difference in surface structure that prevents adsorption of the bacteriophage *(8, 14)*. Complementarity of the surface structures of the bacteriophage and the resistant bacteria may be restored by an independent mutational change in the bacteriophage *(7)*.

The bacteriophage sensitivities and resistances of pure cultures have been considered stable characteristics. Without the advantage resulting from elimination of sensitive organisms by bacteriophage action, resistant mutants usually have little chance in competing against the predominant sensitive cells. Novick and Szilard *(12)* studied sporadic cycles in the numbers of resistant mutants in a culture of *Escherichia coli* in the absence of bacteriophage action. Decreases in proportion of resistant bacteria were ascribed to displacement of the entire bacterial population by a new sensitive population arising from adaptive, sensitive mutants. Increases in proportion of resistant bacteria were ascribed to mutations to resistance in each new sensitive population. Such periodic replacement of a bacterial population by a new sensitive type having selective advantage over the previous type has been substantiated as a mechanism that postpones the establishment of mutational equilibrium *(1)*.

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METHODS

Culture sources and propagation procedure. Pure cultures of lactic streptococci were isolated from cultures obtained from the Dairy Research Institute of New Zealand, from commercial cultures, and from raw milk. Only cultures that coagulated skimmilk rapidly were studied. These were propagated daily (six days per week) in skimmilk fortified with 0.75% nonfat milk solids and autoclaved at 121° C. for 13 min. The inoculum was five drops of culture per 33 ml. of fortified skimmilk. Inoculated bottles were incubated at 22° C. for 16 hr. and then refrigerated. Skimmilk was examined for inhibitors by a test using *Streptococcus thermophilus* and *Streptococcus cremoris (5)*. Skimmilk that contained inhibitors was not used.

Procedure for determining bacteriophage sensitivity. Tubes of litmus milk inoculated with two drops of the desired bacteriophage filtrates, and one control tube, were inoculated with one drop of fresh culture. The tubes were incubated at 32° C. for about 8 hr. and compared soon after the control tube had coagulated. Bacteriophages that did not permit typical coagulation were considered active.

Procedure for determining adsorption of bacteriophages. A sample of bacteria was grown at 32° C. for 2 hr. in sterile skimmilk. Aliquots of bacteria and bacteriophage filtrate were transferred to a tube of sterile skimmilk that was preheated to 32° C. After 14 min. had been allowed for adsorption, the mixture was centrifuged for 8 min. at 5,700-5,900 r.p.m. in a Servall, Model XL, angle centrifuge and the supernatant assayed immediately for unadsorbed bacteriophage particles. The counts of bacteria present in the adsorption mixtures were from 3.5×10^7 to 1.2×10^8 per milliliter. The bacteriophage counts in the mixtures were from 5.0×10^6 to 2.0×10^7 per milliliter.

Enumeration procedures. Bacteria were enumerated by plate count on modified trypticase soy agar *(9)*. Bacteriophages were enumerated by plaque count *(13)*.

RESULTS

Changes in sensitivity to bacteriophages. Approximately 20 cultures, purified by colony isolation, had been propagated daily for about 4 mo., when it was observed that three originally resistant isolates were now susceptible to Bacteriophage 20a. Finding this, the sensitivities of the cultures were rechecked, disclosing that 11 of the cultures had sensitivities different from those observed initially. During the following 6 mo. the remaining cultures and others isolated from changed cultures were checked weekly. Seven additional changes in sensitivity were observed. Only five cultures, about one-fourth of those studied, did not change. Some of the cultures produced lactic acid slightly more rapidly after they changed.

After the changes had been observed, the activities of the bacteriophages were checked using unchanged cultures. The results revealed that the activities of the bacteriophages had not changed during the course of the experiment.

The cultures used for checking the bacteriophages were some that had been kept frozen and some that were obtained from the original sources.

All of the cultures except ML3 were classified as *S. cremoris*, on the basis of neither growing at 40° C. nor producing ammonia from L-arginine. Culture ML3 was classified as *S. lactis* before and after it became resistant to Bacteriophage m13.

TABLE 1

Summary of the changes observed in the sensitivity of lactic streptococci to bacteriophages

Culture	Source	Bacteriophage sensitivity	
		Before change	After change
4B	Commercial culture	4b	4b[a] + 20a[a, b]
4B	Commercial culture	4b	20a
4B1	4B	4b	4b + ab3
4B2	4B	4b	Resistant
KH2	KH	kh	kh[a] + kha[a]
KH2	KH	kh	Resistant
KH3	KH	kh	kh + 20a[a]
M9B	Commercial culture	kh	kh + ab3
ML3	ML3	m13	Resistant
K1	K[c]	kha	kha[a, d] + e8[a, d]
TR	TR	tr	20a[d]
R1	R1	r1	20a[d]
R6	R6	r6	20a
E8	E8	e8	20a[d]
M9C	Commercial culture	15c	20a[d]
A	Milk	Resistant	20a
B	Milk	Resistant	20a
C	Milk	Resistant	20a

[a] Cultures sensitive to this bacteriophage were isolated from the changed culture.

[b] Bacteriophage 20a was isolated from cottage-cheese whey, using a culture of *Streptococcus cremoris* that had been isolated from a commercial culture. This culture was discarded and Bacteriophage 20a was purified and thereafter propagated on Culture 20A, which had been isolated from milk.

[c] This culture, labeled K, was received from a commercial source. Upon arrival it was a mixture of bacteria, some sensitive to Bacteriophage e8 and some sensitive to kha.

[d] Enumeration of this bacteriophage by the limiting active dilution method with the changed culture gave the same result as enumeration with the homologous host.

The changes in sensitivity that were observed are listed (Table 1). They can be divided into the following four types:

1. In six of the 18 changes, cultures became resistant to their homologous bacteriophages. Simultaneously, resistance to Bacteriophage 20a changed to sensitivity. Four of these cultures were lysed by a filtrate of Bacteriophage 20a when it was diluted $1:10^9$ with litmus milk, indicating that the activity of the bacteriophage was multiplication rather than mere inhibition *(2, 10, 17)*. The other two cultures were not used in checking activity of the diluted filtrate.

2. In three of the changes, cultures that had been resistant to all of the available bacteriophages became sensitive to Bacteriophage 20a. These changes were similar to those of the first type, except for the original resistance of these cultures.

3. In the third type of change, single-sensitivity bacteria became a mixture with two sensitivities, requiring two bacteriophages to stop acid production.

Both types of bacteria could be isolated. Six of the 18 changes were of this type. The difference from the first type might have been merely that the less competitive bacteria had not yet been overgrown. Two examples follow:

(a) Culture 4B. Some activity was indicated for Bacteriophage 4b by the fact that Culture 4B coagulated weakly when inoculated with the bacteriophage. The weakly coagulated culture was plated and two colonies isolated. These were resistant to Bacteriophage 4b and susceptible to 20a. We then found that a mixture of Bacteriophages 4b and 20a lysed Culture 4B.

Culture 4B (not inoculated with bacteriophage) was plated and 12 isolates were found susceptible to Bacteriophage 4b. Three were retained. After 5 wk. one of these cultures changed. A mixture of Bacteriophages 4b and ab3 was then required to stop acid production.

(b) Culture KH. Three cultures were checked for changes in susceptibility to Bacteriophage kh. KH2 and KH3 had been isolated from the original culture; M9B had been isolated from a commercial culture.

KH2 was now questionably lysed by the unpurified mixture of wheys, and appeared resistant to Bacteriophage kh. This culture was plated and an isolate chosen that was sensitive to the mixture of wheys. This isolate, KHA, was used in isolating an active bacteriophage from the mixture of wheys. Since the resulting bacteriophage only partially lysed KH2, ten resistant cultures were isolated by inoculating KH2 with the bacteriophage and then permitting coagulation and plating the coagulum. All of these resistant cultures were lysed by the original kh phage; none was lysed by the new bacteriophage. It was thereafter determined that a mixture of Bacteriophages kh and kha lysed Culture KH2.

KH3 now was only questionably lysed by kh. Two resistant isolates from this culture, prepared by plating KH3 after coagulation in the presence of kh, were resistant to Bacteriophages kh and kha; they were sensitive to 20a. A mixture of kh and 20a stopped the production of acid by KH3.

M9B remained sensitive to Bacteriophage kh until 2 mo. after the first recheck. It then appeared resistant and required a mixture of Bacteriophages kh and ab3 to stop the production of acid.

4. The fourth type of change occurred in three cultures. These cultures became resistant to their homologous bacteriophages and then were resistant to a mixture containing all the available bacteriophages. The difference from the first type may have been only that bacteriophages active against the resistant cultures were not available.

Methods of avoiding changes in sensitivity. In rechecking sensitivities of the cultures at weekly intervals, it was observed that cultures propagated every other day changed less frequently than cultures propagated daily and that any changes occurred gradually, after they were detectable, over a period of several days to 3 or 4 wk. Cultures plated when they first appeared resistant to their homologous bacteriophages usually yielded sensitive isolates.

Two methods were used to eliminate the need for periodic isolations. The first involved maintaining an extra isolate or propagation of each culture. When one of the cultures developed resistance to the homologous bacteriophage, it was discarded and two propagations were started from the other culture. The second method involved maintaining quantities of lyophilized cultures under refrigeration. Fresh cultures could be started when changes occurred in those being propagated.

Adsorption studies. Adsorption experiments were conducted with several of the cultures and bacteriophages. The results (Table 2) indicate that alterations in adsorption characteristics (assumed to reflect changes in surface structure) occurred simultaneously with changes in sensitivity. The active homologous bacteriophage was readily adsorbed to the cells of a sensitive culture. When a culture became resistant, that bacteriophage was no longer adsorbed. When a culture became sensitive, the bacteriophage that was then active was readily adsorbed, though it had not been before the change.

TABLE 2

Differences in the adsorption of bacteriophages before and after changes in sensitivity in cultures of lactic streptococci

Culture	Source	Sensitivity	Bacteriophage added	Per cent adsorption
KH2S	KH2	kh	kh	97
KH2R	KH2	Resistant	kh	0
ML3	ML3	m13	m13	95
ML3R	ML3	Resistant	m13	0
K1S	K1	kha	kha	86
K1R	K1	e8	kha	0
K1S	K1	kha	e8	0
K1R	K1	e8	e8	97
20A	Milk	20a or c1	c1[a]	93
M9C	Commercial culture	15e	c1	0
M9CR	M9C	20a or c1	c1	90
R1	R1	r1[b]	r1	93
R1R	R1	20a or c1[b]	r1	91
R1	R1	r1	c1	39
R1R	R1	20a or c1	c1	48

[a] Bacteriophage c1 rather than 20a was chosen for adsorption studies because 20a did not produce countable plaques.

[b] Bacteriophage 20a or c1 could inhibit R1; Bacteriophage r1 could inhibit R1R.

One exceptional case—in Cultures R1 and R1R—involved little or no change in the adsorption of bacteriophages. Before a change in sensitivity, Bacteriophage c1 or 20a (which acted the same) inhibited Culture R1; after the change, Bacteriophage r1 inhibited the changed culture. Yet neither of these infections resulted in bacteriophage multiplication. Other reports have described inhibition without bacteriophage multiplication *(2, 10, 17)* and slow adsorption with bacteriophage multiplication *(4)*.

DISCUSSION

The results, together with the findings of Luria and Delbruck *(8)*, Novick and Szilard *(12)*, and Atwood *et al. (1)*, suggest an explanation of the changes

in sensitivity that occurred in the absence of bacteriophage action. Resistant mutants *(8)* and adaptive mutants *(1, 12)* normally occur as independent mutations in separate bacterial cells in the predominant (sensitive) component of a bacterial population. This does not rule out the possibility that there may be an occasional adaptive mutant with altered surface structure. Such a mutant would be expected to develop into a resistant population that displaces the sensitive population. That such mutations did occur in these cultures of lactic streptococci is indicated by the types of change observed, the methods that prevented changes in sensitivity, and the adsorption behavior of the bacteriophages.

A possibility that cross contamination of cultures, followed by growth and domination, could result in changes similar to those observed has been considered. The following points indicate that the above explanation is more tenable: *(a)* Cross contamination is less likely than contamination with bacteriophages, particularly in a laboratory where bacteriophages are cultured. There were no sudden failures in acid production, indicating that the cultures were adequately protected against bacteriophage contamination. *(b)* Changes occurred most frequently in two very fast acid-producing cultures, KH and 4B. *(c)* Culture R1 adsorbed Bacteriophage r1 readily and c1 slowly, before and after the change in sensitivity. *(d)* The only *S. lactis* culture, ML3, was classified as *S. lactis* after the change in sensitivity. *(e)* Culture C2 (obtained after the above experiments were completed) changed so as to exhibit resistance toward only one of three originally homologous bacteriophages. *(f)* None of the cultures in the study before the isolation of KHA was sensitive to Bacteriophage kha.

Altered adsorption characteristics were apparently not involved in a change in Culture R1 that enabled multiplication of Bacteriophage c1 (or 20a) and prevented multiplication of Bacteriophage r1. This case serves to emphasize that bacteriophage multiplication does not always result when particles of a bacteriophage are adsorbed to the cells of a culture. However, it appears that, in most cases, the factor preventing bacteriophage multiplication is adsorption failure.

Cultures of lactic streptococci that are not known to have been acted on by bacteriophages—though bacteriophages active against them are often known—generally are referred to as primary growth; whereas, cultures that are known to have arisen after lysis of sensitive cells by a bacteriophage are referred to as secondary, or resistant, bacteria. Secondary bacteria have often been found to be sensitive to bacteriophages other than those used in their isolation *(11, 16, 18)*. In the present study, where resistance apparently was gained by natural selection of mutants, several of the resulting cultures were sensitive to other bacteriophages. This suggests that there is not a significant difference between primary and secondary, except as the terms might apply to the relationship between a culture and a particular bacteriophage—in which case the term sensitive or resistant will usually suffice.

Differences between a resistant population naturally selected and one artificially selected (by lysis of sensitive cells) can be the result of one or both of two differences between the mechanisms involved. Without bacteriophage

action, a rare resistant mutant must have some other competitive advantage—at least a small one—to be able to displace a sensitive population. On the other hand, when the sensitive cells in a culture are lysed, a resistant culture may arise from any viable resistant bacterium, regardless of competitive ability. In the former case, the resulting cultures might be more stable. Secondly, when a bacteriophage is employed in the isolation of resistant cultures, there is a possibility that the resulting cultures will be lysogenic *(15)* or carriers of the bacteriophage *(6)*.

Hunter *(6)* suggested that the constant action in nature of a variety of bacteriophages on lactic streptococci accounts for some of the differences in sensitivity. On the other hand, the present results suggest that, when cultures multiply for long periods in the absence of bacteriophage action, differences in sensitivity tend to disappear as a result of natural selection. This could account for the comparatively few different bacterial strains or types (as judged by difference in bacteriophage sensitivity) that were found in an examination of 14 commercially used cultures *(3)*.

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CONTINUOUS AND AUTOMATIC PROPAGATION OF DAIRY CULTURES[1]

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SUMMARY

A small fermentation system was devised for continuous automatic propagation of dairy cultures. The fermentor is a circulatory, 1-in. diameter, stainless steel pipe assembly of 1,000-ml. capacity, in which skimmilk is continuously and slowly circulated. Acidity is controlled with a pH meter and meter-relay which activates a solenoid valve controlling the skimmilk inflow. The fermentation system has been operated for 12 wk., but not without contamination and undesirable fermentations. Successful trials free from contamination have been conducted for 3 wk. These have provided information regarding the throughput rate at various temperatures between 50 and 90° F. At 90° F. the retention time or growth rate was 30 min. The critical pH level was 5.3, below which the rate of growth was reduced. Dairy cultures can be propagated continuously at maximum growth rates without impairment of flavor or activity, with constant temperature and pH (above 5.3 for maximum rate) and in continuous motion.

Many industrial fermentation processes today are operated continuously, automatically. Continuous fermentations have been investigated and applied, with varying degrees of success, to the production of antibiotics, enzymes, acetic acid, casein, fatty acids and their esters, lactic acid, industrial alcohol, bakers' and food yeast, solvents, sulfur, and vinegar. When continuous fermentations are applied successfully to commercial operations, the advantages obtained are space and labor savings, as well as quality improvement and greater yield of the product.

Reviews *(11, 16)*, theoretical treatments *(4, 7)*, and other publications on various related techniques and processes *(1, 2, 6, 8, 10)* are of value in obtaining an understanding of the techniques and current research in this rapidly growing field.

In the field of dairy science, commercially successful, continuous fermentation processes are used in the production of lactic acid and in the manufacture of commercial casein *(15, 18)*. For lactic acid production, Whittier and Rogers *(17)* maintained cultures of lactobacilli and streptococci at 43° C. between pH 5.0 and 5.8 by periodic additions of calcium carbonate to fermenting whey. Kempe *et al. (5)* and Finn *et al. (3)* have reported results of studies of continuously controlled pH on lactic acid production by *Lactobacillus delbruckii* at various levels between 3.5 and 6.0. Meyer *(9)* reported successful automatic cream neutralization for butter manufacture, using electronic pH control.

The purpose of this study was to assemble the equipment needed for suitable control of continuous fermentations, and to investigate the fundamental bacteriological principles involved during continuous growth or propagation of lactic

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acid–producing dairy cultures, used in the manufacture of buttermilk and various kinds of cheeses. It was believed that the information obtained would be of value in the development and application of continuous, automatic methods of processing the various fermented dairy products.[2]

EQUIPMENT AND APPARATUS

To aid others who might be interested in further work along these lines, a detailed description of the apparatus and equipment used is given.

Figure 1 schematically illustrates the apparatus design and controls. The internal capacity of the circulatory pipe system was 1,000 ml.

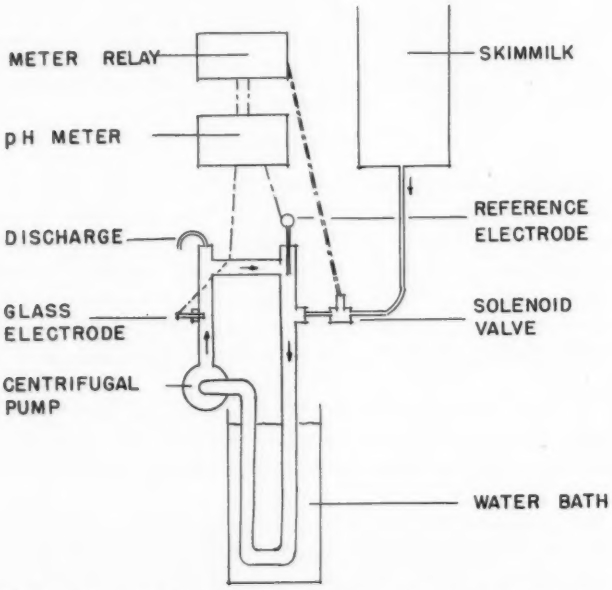

FIG. 1. Schematic illustration of fermentation system and controls.

A Beckman Model H-2 pH meter was used. The manufacturer recommends that this model not be used in grounded electrode systems such as flow lines, but reasonably satisfactory results were obtained under the conditions described here. The various components were grounded as required. General purpose glass electrodes (Model 8990-80) were used. Sleeve-type reference electrodes (Model No. 8970-14) were found to give better results than those of the fiber junction type.

[2] Recently another paper has appeared on this subject, W. J. HARPER AND D. A. SEIBERLING, Method and Apparatus for the Continuous Production of Cheese-Forming Curd. U. S. Patent 2,781,269. *J. Dairy Sci.*, **40**: A30. 1957.

The meter-relay was obtained from Assembly Products, Inc., Chesterland, Ohio. It is known as a single-contact, automatic-reset Versatrol, Catalog No. 3581, with Model 451-C single-contact high limit. Its range is 0 to 1 milliampere DC, with a 2-sec. interval timer. Using the pH meter reading, which was synchronized with the scale on the meter-relay, it was possible to select a desired pH and manually set the contacts at the desired pH on the meter-relay for automatic pH control. The output of the meter-relay operated at 110-volt, ¼-in. solenoid valve on and off as required to maintain constant pH.

The circulation pump was a Tri-Clover 1-in. milk pump (Type MS—No. 2075, patented January 27, 1931), which was salvaged for this study. The impeller was replaced with a new blade, approximately 1½ in. long, which reduced the rate of flow to 2½ gal. per minute at 1,725 r.p.m.

The entire assembly was made with standard 1-in. stainless steel milk piping and fittings. It was so designed that the entire system—including the pump—could be autoclaved after assembly. The electrodes were sterilized by alcohol immersion and aseptically attached after autoclaving the system. This procedure has its limitations and might have been a source of contamination. The saturated KCl was not sterilized and also may have been a source of contamination.

When in operation, the immersion of part of the fermentation pipe system into a water bath equipped with heater, stirrer, and thermostat was satisfactory for accurate temperature control.

METHODS

For total bacteria counts, the regular standard plate count procedures were used with Plate Count Agar (Difco), M-P-H Medium (BBL), or Tomato Juice Agar (Difco). For coliform counts, violet red bile agar medium was used.

The creatine test was used to aid in evaluating flavor quality, but in general it was found that organoleptic examination by qualified judges was the most satisfactory manner of judging the quality of the product.

All skimmilk used was reconstituted to give a final serum solids concentration of 10.5 %. The skimmilk was sterilized by autoclaving in quart glass containers approximately three-fourths full for 16 min. at 15 lb. of pressure. The sterile milk was then aseptically transferred to the skimmilk storage tank of the fermentation system and held at 40° F.

PROCEDURE

All trials were conducted using regular commercial lactic streptococci dairy starter cultures. In some instances, the fermentation system was inoculated with fresh liquid cultures which had been propagated in the usual manner. In other instances, the fermentation system was inoculated by simply pouring in powdered cultures.

When in operation, the skimmilk supply tank was located at a higher point than the discharge outlet of the fermentation system. This permitted the circulator system to remain completely full. Upon actuation of the solenoid valve, the fermentation discharged by overflowing an amount of product equal to that

permitted to flow into the system. The inflow continued until the pH level was raised approximately 0.2 to 0.3 unit.

It was possible to cause the input to become relatively uniform by throttling the flow through the rubber hose with a screw clamp, but difficulties then were encountered due to occasional plugging of the system with particles of coagulated milk. The most successful operation was obtained when the flow was adjusted to permit about 150 to 200 ml. of skimmilk to enter at one time over a period of a few minutes and then to stop for from 10 to 30 min., depending upon the throughput rate and temperature of operation.

RESULTS

The fermentation system has been operated continuously for periods of up to 12 wk., but not without some difficulties of contamination and undesirable fermentations. Approximtely one-half of all trials failed—owing to contamination within the first 24 hr.—even when performed with the greatest of care. Considerable improvement of techniques or apparatus is needed for the system to prove of practical value. However, it is significant that some trials were successful, which is the first evidence to that effect. Successful trials (free from contamination) of from 2 to 3 wk. duration have been conducted. These have provided a better understanding of the cultural and biochemical characteristics, under conditions of continuous growth and in constant slow motion or agitation by circulation in the circulatory pipe system.

During these studies, all acidities were determined by both titration and pH. The relationship of titratable acidities and pH is shown (Figure 2). There was always good agreement of the two methods. The solids content was maintained at a constant level of 10.5%.

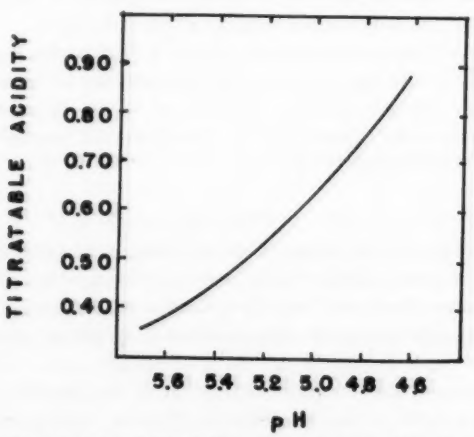

FIG. 2. Relationship of pH and titratable acidity in reconstituted nonfat milk solids at 10.5% concentration.

An important consideration of a continuous fermentation system is the throughput rate, or the rate of flow per volume of fermentor. The holdup, or retention, time is calculated as one over the throughput rate per hour. For all practical purposes, the retention time as used here may be considered equal to the growth rate, or generation time, of the microorganisms present.

When the fermentor was set at a given pH and constant temperature and allowed to flow at the rate influenced by these factors, the total count remained reasonably constant at the level indicated. Total microorganism plate counts at the various pH levels are shown (Figure 3).

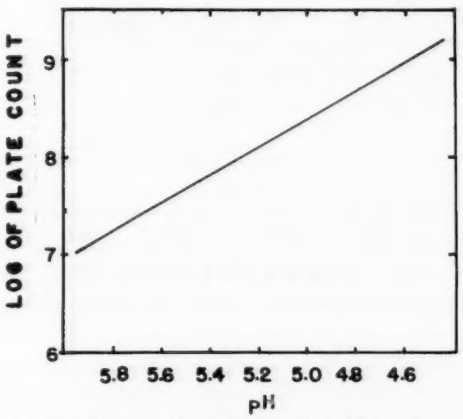

FIG. 3. Influence of pH on the total plate counts.

Figure 4 shows the retention time at various temperatures between 50 and 90° F. at pH 5.5. The curve shows that, as the temperature increased, the retention time decreased significantly. This indicated an increase in the growth rate of the organisms present.

Influence of pH on the retention time is shown (Figure 5). As the acidity increased, the presence of greater amounts of lactic acid tended to reduce the rate of organism growth, although the total count was higher (as shown in Figure 3). There also was greater reduction in rate at the lower temperature of 68 than at 80° F. The reason for this difference was not apparent, but may have been simply individual strain differences between the two cultures used in obtaining these data.

DISCUSSION

The generation time at 90° F. for the dairy starter cultures used was 30 min. Comparing this with other data, Powell *(14)* showed that the generation time of individual bacteria varied widely. He reported generation times of from 6 to 50 min. for *Streptococcus faecalis.* Politi *(13)* also observed "that considerable differences can be had from one strain to another, especially in an advanced stage of growth." He reported that the maximum multiplication rate for *Strep-*

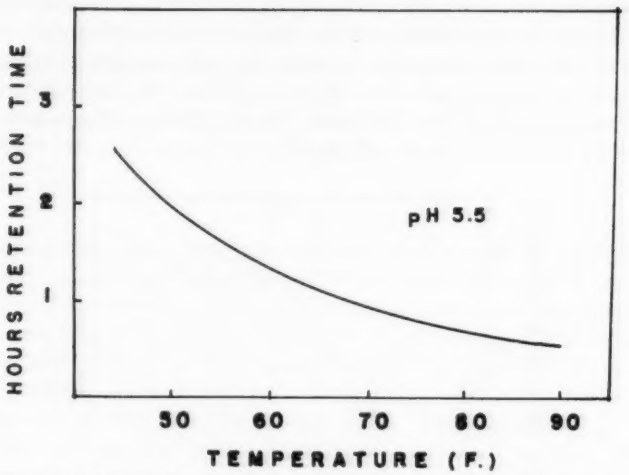

FIG. 4. Influence of temperature on the retention time of the cultures.

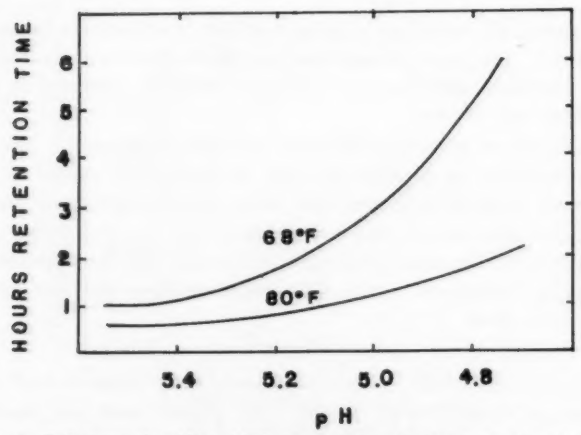

FIG. 5. Influence of acidity (pH) on the retention time of the cultures.

tococcus lactis at 34° C. was 30 min. Herbert *et al. (4)* reported that the doubling time of *Aerobacter cloacae* was 49 min. The generation times of the starter cultures reported here are in general agreement with other organisms. This showed there was no significant difference in growth rate under conditions of slow agitation and continuous propagation.

The limiting pH level seems to be somewhat higher under conditions of continuous growth than in single-growth cycle batches. Overcast *(12)* showed that pH 5.0 was almost the limiting level for several strains of *S. lactis* used in an excellent study of the influence of pH on the proliferation of lactic streptococcus bacteriophage. In all of the trials reported here, the first measurable indication of a reduction in growth took place at pH 5.3, with greater reductions as the pH was lowered. Longsworth and MacInnes *(6)* and Kempe *et al. (5)* showed that careful control of pH at the optimum of 5.8 to 6.0 resulted in more complete fermentation at faster rates in the production of lactic acid by *Lactobacillus acidophilus.*

As the pH level lowered, the first signs of visible coagulation occurred at pH 5.0 to 4.9. The maintenance of satisfactory body and texture characteristics is essential in dairy products, if not in starters. When the fermenting skimmilk was removed from the fermentor above pH 5.3 and allowed to remain motionless, normal coagulation occurred. Preliminary evidence indicates that such coagulation can take place while the products is in very slow motion through a stationary pipe system.

The conditions under which suitable strain balance occurs in mixed cultures have not been established. When mixed cultures were used, the desired aroma slowly disappeared over a period of from seven to ten days, as measured by organoleptic tests. At the beginning, the flavor and aroma were excellent, but they progressively decreased until only a clean acid flavor was detectable. In other trials, with single-strain lactic streptococcus cultures, the flavor remained uniformly satisfactory until contamination occurred. When contamination occurred, off-flavors resulted, as well as reduction in starter activity.

It appears that the application of continuous fermentation to commercial dairy operations would be of considerable value and has future possibilities. Based on the information available, it is possible to speculate that continuous automatic methods of processing the various dairy products will prove feasible. This work does establish certain fundamentals and shows the need for further investigation.

ACKNOWLEDGMENTS

We deeply appreciate the technical assistance of Orlean Clarke and Dudley Thomas.

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ELECTRONIC MICROSCOPIC OBSERVATIONS OF THE STRUCTURE OF CHEESE

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SUMMARY

This study was initiated to develop a method for the examination of the ultra-fine structure of cheese with the electron microscope. It was used to study the structure of Cheddar cheese made from variously treated milk. Freshly broken surfaces of chilled Cheddar cheese were covered with a parlodion film. Next, the film was stripped and the replica side covered with a formvar film. After dissolving the parlodion portion, the formvar film was shadow-cast with gold and examined under the electron microscope.

Observations made on replica films showed specific surface patterns for cheese made from raw, pasteurized, and pasteurized homogenized milk.

At least three groups of workers *(3, 4, 7)* have reported on the use of the electron microscope in the direct examination of ultra-thin slices of cheese. Two of the reports *(3, 4)* show no illustrations; whereas, the third report *(7)* shows illustrations that are difficult to interpret. According to Leber *(4)*, the slicing of cheese into sections of 0.3 μ or less, and the transfer of these ultra-thin slices to a film or grid, are some of the problems in using this method.

Another method, investigated by Imhof and Hostettler *(3)*, is the replica, or indirect, method. Difficulties in obtaining a replica film that would strip properly caused them to abandon this method.

This paper deals with the development of a two-step replica method *(1)* and its use in the indirect examination of the ultra-fine structure of cheese made from raw, pasteurized, and pasteurized, homogenized milk.

EXPERIMENTAL PROCEDURE

Representative portions of cheese were cut with a wire cutter into strips of approximately 5 by 5 by 50 mm. These strips were grouped, wrapped in tinfoil, and chilled for 4 hr. or longer in the ice compartment of a refrigerator. The chilled strips were carefully broken by hand and suitable sections selected for further work. Broken surfaces that were relatively level, and without areas originating from openings within the cheese, were covered immediately with a thin layer of chilled parlodion solution, kept in ice water. The solution consisted of 2 g. parlodion dissolved in a mixture of 0.05 ml. amyl acetate, 20 ml. absolute ethanol, and 80 ml. ethyl ether. The composition of this solution was developed in the early part of this study. It contained a minimum of ethanol necessary to dissolve the parlodion in ether. The small amount of amyl acetate

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served to prevent curling of the stripped film. Higher concentrations of amyl acetate caused strong adhesion of film to cheese and made stripping difficult or impossible.

After depositing the parlodion solution with an eye dropper, in quantities sufficient to cover the selected surface, the section was placed on clean paper in a manner to permit drainage of excess fluid. As soon as the film showed signs of dryness, a second application of parlodion solution was made. This was repeated five times, or until a film of sufficient thickness and strength was obtained that could be stripped without tearing or breaking. The film, while still on the cheese, was trimmed to size with a sharp scalpel. The trimmed film was stripped and placed in chloroform with replica-side up. This step was introduced to wash off loose cheese particles, as well as to remove any possible fat adhering to the film.

The washed film was placed on a clean glass slide with replica-side up and covered with a solution of 0.125% formvar in chloroform *(1)*. As soon as the formvar had set, the film was transferred to an electron microscope grid with the formvar-side down and immersed into a 1:1 ethanol:ether mixture, to dissolve the parlodion film. This required about 15 min. Next, the grid and formvar film with the replica-side up were removed from the solvent, air-dried, gold-shadowed in a Radio Corporation of America shadow-caster Model EMV-6 at an angle of 15°, and examined under a Radio Corporation of America electron microscope Model EMU-2D.

Pictures were taken of the most commonly observed types of replica structure of cheese on a given film, or several films, obtained from the same lot of cheese. Best pictures were obtained at magnifications of from 5,000 to 10,000.

RESULTS

The cheese examined in this study was taken from experimental lots made in connection with another project. The 6-mo.-old Cheddar cheese examined in 1955 was made from unstandardized Holstein milk. Additional series of 6-mo.-old cheese made from Holstein and standardized Jersey milk were examined in 1956. All cheese was made by the same method *(5)*.

More than 200 replica films were examined under the electron microscope and a total of 75 pictures taken. A representative series of photographs, obtained from replica films of Holstein milk cheese in 1955, is shown (Figure 1). In general, pictures taken in 1956 from Holstein and Jersey milk cheese were rather similar in pattern to those shown in Figure 1.

A close examination reveals certain differences in the surface patterns of the four types of cheese. The differences shown were observed repeatedly, and are regarded as differences in the ultra-fine structure of cheese made from variously treated milks. The raw-milk cheese *(a)* shows a surface of volcanic appearance, with long fibers extending to a considerable height above the general surface of the cheese. Such fibers are not evident in the other three types of cheese. A possible explanation for this difference may be the more

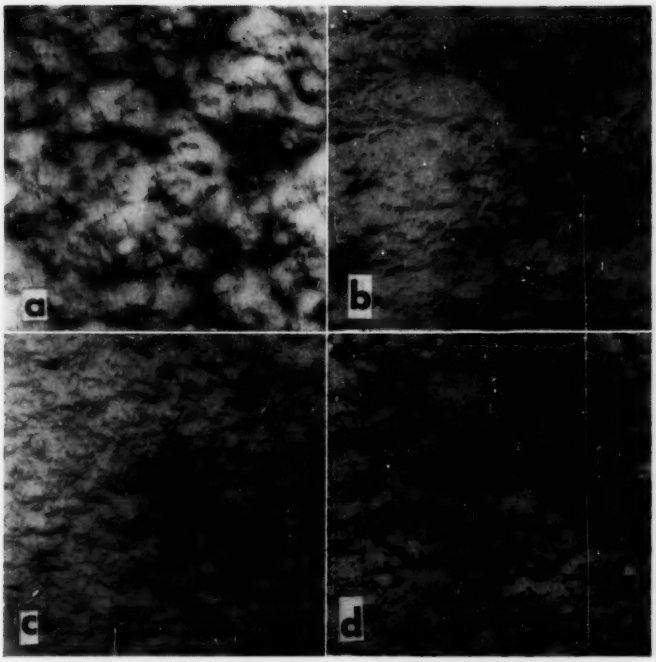

FIG. 1. Electron microscope photographs of surface replica of a series of Holstein milk cheese made from *(a)* raw, *(b)* pasteurized, *(c)* pasteurized, homogenized at 1,000 p.s.i., and *(d)* pasteurized, homogenized at 2,000 p.s.i. Magnification 21,000 ×.

elastic body observed in raw-milk cheese. The pasteurized milk cheese *(b)* differs in surface patterns from the two pasteurized, homogenized milk cheeses *(c, d)*. This may be owing to the fact that homogenization changes the physical structure of casein *(2)* and progressively reduces the elastic properties of cheese *(5)*. That homogenization must be considered an important factor is evidenced by the greater difference in surface patterns between pictures *(b)* and *(d)* than between *(b)* and *(c)*, picture *(c)* representing cheese made from milk homogenized at 1,000 p.s.i. and *(d)* at 2,000 p.s.i.

DISCUSSION

Considering the limitations of the electron microscope, in that only ultrathin preparations lend themselves to examination in a vacuum under the bombardment of electrons, it seems that a replica method, such as developed in this study, has considerable merit. Although Imhof and Hostettler *(3)* have published a method recently by which defatted cheese, hardened with ethanol and formaldehyde, may be embedded and cut successfully into slices of 0.1 μ or less, the method is more involved and at best shows the structure of cheese only after

treatment with several reagents. Since Imhof and Hostettler did not show any pictures, no comparison is possible at this time.

In breaking the chilled cheese by hand, it was reasoned that the break would occur in all instances at the weakest points in the area under stress. Breaking the cheese would also eliminate such distortion of the broken surface pattern as might take place by mechanical cutting. However, no comparison was made to prove or disprove the above assumption.

The application of chilled parlodion solution on chilled cheese was used to produce the minimum interaction between the solvents and the cheese constituents, thereby obtaining as true a replica as possible of the freshly broken cheese surface. The gradual build-up of the parlodion film, as the first replica, was essential in obtaining a film that would dry uniformly on the surface of the cheese and produce the most nearly identical replica.

Studies conducted thus far have been with aged Cheddar cheese only. The above method may prove useful in observing possible structural changes of curd during ripening. Other possible applications are comparison studies of structures of acid- and rennet-type curd of natural and reconstituted milk curd, and many others.

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EFFECTS OF REDUCING RENNET AND ADDING CALCIUM CHLORIDE ON THE MANUFACTURE AND CURING OF CHEDDAR CHEESE[1,2]

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SUMMARY

This study showed the lack of real benefits derived from using low levels of rennet with added calcium chloride in the manufacture of Cheddar cheese.

Cheese was made from raw and pasteurized milk during four seasons. Each lot of cheese consisted of three vats to which 90, 45, and 45 ml. rennet per 1,000 lb. of milk was added, respectively. The third vat of milk was further treated with 0.02% calcium chloride. The cheese was cured at 50° F. for 36 wk. Using subnormal amounts of rennet delayed curd formation in both raw and pasteurized milk. Adding 0.02% calcium chloride with one-half normal amounts of rennet gave normal curd formation and yield, but protein breakdown was slow, and curdiness persisted longer in the finished cheese. The quality of the cured cheese was not influenced by variations in the rennet and calcium chloride used, but persistence of curdiness was undesirable in young cheese made with reduced levels of rennet, especially when supplemented with calcium chloride.

Adding calcium chloride to milk for cheese-making reduces the amount of rennet needed for optimum curd formation *(12, 17)*, improves curd-making properties under certain conditions *(29)*, and may increase the yield of cheese *(14, 17, 21)*. The use of calcium chloride with less rennet has been advocated by some practical operators for reducing costs. The Federal Definitions and Standards of Identity *(8)* permit the addition of the equivalent of 0.02% anhydrous purified calcium chloride to milk for cheese-making.

It is the purpose of this paper to report the results of using reduced amounts of rennet, with and without calcium chloride, on the manufacture and ripening of Cheddar cheese made from raw and pasteurized milk. The factors observed were: curd firmness at cutting time; recovery of milk solids in the cheese; extent of ripening in terms of proteolysis and fat hydrolysis at 8 and 36 wk. of age, and cheese quality at 2, 8, and 36 wk. after making.

EXPERIMENTAL METHODS

Cheese-making. The milk came from the mixed milk supply of the University of Wisconsin dairy manufacturing laboratory.

Cheese was made in September, December, March, and July, to represent milk from four seasons. Four lots of cheese were made in each of these months:

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[2] This study was supported in part by research grants from Marschall Dairy Laboratories, Madison, Wisconsin, and Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin.

two lots from pasteurized milk and two from raw milk. One extra lot of raw-milk cheese was made in September. Raw milk was made into cheese on the day it was received. Milk which had been pasteurized at 143° F. for 30 min. and cooled to 40° F. was held overnight before manufacturing. Each lot consisted of three vats in which equal amounts of identical milk and culture were used. Vats A, B, and C received 90, 45, and 45 ml. of rennet, respectively, per 1,000 lb. of milk. Calcium chloride was added at the rate of 0.02% of the milk to Vat C. These vats will be referred to in this discussion as Treatments A, B, and C. Fresh commercial rennet and calcium chloride of analytical reagent grade were used. Calcium chloride to be added to the milk was dissolved in distilled water at the rate of 90.8 g. anhydrous calcium chloride per liter. The interval between adding rennet and cutting the curd was standardized at 30 min. for all vats, in order to measure the clotting effects of each treatment.

All vats of milk were made into cheese by a routine procedure *(22)*. After milling, the curd was sprayed with approximately 25% of its weight of water at 90° F., and was forked intermittently and completely drained before salting. The cheese was formed into 10-lb. blocks, wrapped in Parakote, and cured at 50° F. for 36 wk.

Fat and dry matter. Fat in milk was measured by the Babcock method, and casein was estimated by formol titration *(9)*. Milk solids were measured by drying 2 g. of milk in 50-ml. beakers containing clean, dry sand. Samples were dried at 110° C. for 16 hr. in a forced-draft oven. Whey was sampled at dipping and tested for fat by the American Association method *(10)*, and for solids by drying 2 g. in covered aluminum dishes for 16 hr. at 110° C. in a forced-draft oven. Cheese solids were measured by drying 2 g. in 50-ml. beakers at 110° C. for 16 hr. *(23)*.

Curd firmness. After rennet had been added and mixed with the milk, duplicate 400-ml. beakers were filled from each vat. Each beaker was suspended in the vat in such a way that the surface of the milk in the beaker was at or below the surface of the milk in the vat. At the time of cutting, 30 min. after adding rennet, curd firmness was measured on the curd in the two beakers by using the official curd knife *(6)* and the apparatus of Sommer and Matsen *(27)*.

Measurement of ripening. Rate of protein decomposition in the cheese was estimated at 8 and 36 wk. by measuring total nitrogen in the cheese, and the per cent of total nitrogen soluble in a water solution of sodium citrate at pH 4.7. All tests were made in duplicate. A modification of Sommer's *(26)* method was used for extracting soluble nitrogen. A sample of cheese weighing 25 g. was placed in a Waring Blendor jar with 5 g. of C.P. sodium citrate and 170 ml. of distilled water, and mixed at high speed for 15 min. The mixture was quantitatively transferred to a 600-ml. beaker, acidified to pH 4.7 with 2 *N* acetic acid, and heated to 50° C. in a water bath. Meanwhile, a filter mat was prepared in a Buchner funnel. Two sheets of Reeve Angel No. 202 filter paper formed the bottom of the mat. A mixture of J. M. Celite and water was poured on the filter paper and compacted with suction. Two more sheets of filter paper were placed over the Celite and the entire mat rinsed with distilled water. The

sample at 50° C. was filtered through the prepared Buchner funnel with gentle suction. The residue was washed twice with small amounts of distilled water at 50° C. and refiltered after each washing. The combined filtrates were then made to a volume of 500 ml. Nitrogen in the cheese and in the acidified sodium citrate extract was measured by the Kjeldahl-Wilfarth-Gunning method *(2)* with the boric acid modification *(18)*.

An estimate of free fatty acids in the cheese at 8 and 36 wk. of age was made by a method developed by Kielsmeier and Swanson *(13)*.

Cheese quality. Each cheese was scored at 2, 8, and 36 wk. of age by three judges who worked independently. Their individual values for each cheese were averaged to give the score of that cheese. The scoring was based on points ranging from 1 to 6, in which 1 was excellent; 2, desirable; 3, satisfactory; 4, objectionable; 5, very objectionable, and 6, unsalable. The judges commonly used one-fourth of a point to distinguish differences.

The first changes in body breakdown were estimated by the curdiness of the cheese, which the judges were asked to consider as a factor separate from body score. This was evaluated by using: 0, indicating no curdiness; 1, slightly curdy; 2, distinctly curdy; 3, very curdy, and 4, extremely curdy.

Cheese flavor intensity, as distinguished from flavor score, was rated by the judges as: 0, possessing no cheese flavor; 1, slight cheese flavor; 2, distinct cheese flavor; 3, pronounced cheese flavor, and 4, extreme cheese flavor.

Statistical treatment of data. Analyses of variance, as outlined by Cochran and Cox *(5)* for randomized blocks, were made on all the data. The significance of differences between treatments in each analysis of variance was determined by the multiple range test described by Duncan *(7)*. Differences were considered statistically significant at the 95% level and highly significant at the 99% level, unless otherwise indicated. Confidence limits at the 95% level of significance were calculated for each mean value presented in the tables. These limits were calculated according to a procedure described by Snedecor *(25)*. Unless otherwise stated, differences or effects of treatments are noted only when they have been found to be statistically significant.

RESULTS

Milk. Average percentages of fat, casein, and total solids in the milk are given (Table 1). Values are normal for this geographical area and indicate a comparable composition of raw and pasteurized milks used.

TABLE 1

Average percentages[a] of fat, casein, and totcl solids in the milk

	Lots	Pasteurized milk	Lots	Raw milk
	(No.)	*(%)*	*(No.)*	*(%)*
Fat	8	3.53 ± .07	9	3.58 ± .09
Casein	8	2.39 ± .08	9	2.45 ± .09
Total solids	8	11.57 ± .27	9	11.69 ± .27

[a] Means and 95% confidence limits.

Curd firmness at cutting. The data (Table 2) show curd firmness measurements made 30 min. after adding rennet. Reducing the amount of rennet decreased curd firmness, but the addition of 0.02% $CaCl_2$ restored the curd firmness characteristic of normal rennet levels. The low curd firmness values

TABLE 2

Effect of reduced quantities of rennet with and without added calcium chloride on curd firmness at time of cutting

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Curd firmness[a]	Lots	Curd firmness[a]
	(ml.)	*(%)*	*(No.)*	*(g.)*	*(No.)*	*(g.)*
A	90	0.00	8	12.0 ± .9	9	15.6 ± .7
B	45	0.00	8	9.8 ± .9	9	12.0 ± .7
C	45	0.02	8	13.0 ± .9	9	15.0 ± .7

[a] Means and 95% confidence limits.

reported (Table 2) for pasteurized milk show the probable influence of pasteurization, and of the cool aging *(1, 24)* which occurred during overnight storage of this milk.

Yield. The data in Table 3 indicate a marked reduction in recovery of milk solids in raw-milk cheese when one-half the normal amount of rennet was

TABLE 3

Effect of rennet and calcium chloride on the proportion of milk solids retained in the cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Milk solids in cheese[a]	Lots	Milk solids in cheese[a]
	(ml.)	*(%)*	*(No.)*	*(%)*	*(No.)*	*(%)*
A	90	0.00	6	50.09 ± .35	9	50.92 ± .30
B	45	0.00	6	49.97 ± .35	9	50.30 ± .30
C	45	0.02	6	50.37 ± .35	9	51.05 ± .30

[a] Means and 95% confidence limits.

used. Yield efficiency was restored by the use of 0.02% calcium chloride in Treatment C. No differences in yield were attributable to Treatments A, B, or C on pasteurized-milk cheese.

Measurement of fat in the whey at dipping showed significantly greater losses in raw-milk whey only following the use of half-normal amounts of rennet. Percentages of fat in whey from raw milk following Treatments A, B, and C were 0.36, 0.39, and 0.35%, respectively, and for pasteurized milk, 0.36, 0.36, and 0.33%, respectively.

Solids-not-fat content of raw wheys were all alike at 6.10%, whereas whey from pasteurized milk following Treatments A, B, and C contained 6.07, 6.09, and 6.10%, respectively.

The explanation of differences in recovery of milk solids observed is suggested by the data (Table 2). Those lots which showed low curd-firmness values tended to have lower recovery of solids in the cheese. The effects of milk composition and cool aging on the yield of cheese solids were not measured, but the combined data in Tables 1, 2, and 3 suggest that lower average casein content and cool aging might have been responsible for soft curd formation which resulted in lower yields in all three treatments of pasteurized milk.

Ripening. Protein decomposition during ripening was significantly affected by the rennet treatments. The data (Table 4) show that at 8 and 36 wk.,

TABLE 4

Effect of rennet and calcium chloride on the soluble nitrogen and extractable fatty acids in cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese			Raw-milk cheese		
			Lots	Soluble nitrogen[a]	Fatty acids[b]	Lots	Soluble nitrogen[a]	Fatty acids[b]
	(ml.)	*(%)*	*(No.)*	*(8 weeks)*		*(No.)*	*(8 weeks)*	
A	90	0.00	8	14.73 ± .34	3.53 ± .09	9	16.47 ± .36	4.03 ± .12
B	45	0.00	8	12.16 ± .34	3.59 ± .09	9	14.22 ± .36	4.08 ± .12
C	45	0.02	8	12.69 ± .34	3.50 ± .09	9	14.32 ± .36	3.99 ± .12
				(36 weeks)			*(36 weeks)*	
A	90	0.00	8	27.21 ± .84	3.59 ± .09	9	30.62 ± .47	5.27 ± .14
B	45	0.00	8	23.91 ± .84	3.80 ± .09	9	27.30 ± .47	5.04 ± .14
C	45	0.02	8	24.10 ± .84	3.58 ± .09	9	27.73 ± .47	5.10 ± .14

[a] Soluble nitrogen as per cent of total nitrogen, showing mean values and 95% confidence limits.

[b] Ml. of 0.05 *N* alcoholic KOH required to neutralize extractable fatty acids from 5 g. of cheese. Mean values and 95% confidence limits are shown.

protein decomposition in the control cheese, Treatment A, was greater than that in cheese made by either Treatments B or C. The difference in soluble nitrogen between the control and experimental cheese, while marked at 8 wk. of curing, was even greater after 36 wk. Amounts of soluble nitrogen in cheese made by Treatments B and C were alike at 8, and again at 36 wk. of age. Proteolytic changes showed the same trends in both pasteurized- and raw-milk cheese. These findings agreed with those of Babel *(3)*.

The conclusion is obvious that the initial changes in normal ripening are definitely associated with rennet action. It seems probable that these initial changes in the protein are associated with the development of smoothness of body. This will be shown by the observations of curdiness (Table 6). Whether these changes in protein are measurably important in flavor development is highly questionable, in view of the flavor scores of the cheese (shown in Table 5).

The fatty acids liberated during curing were not affected by the levels of rennet or by the calcium chloride used.

The data of Table 4, however, show that greater amounts of extractable fatty acids were produced in the raw-milk cheese, which also had the highest flavor intensity. This would be expected from the results of studies by several other workers who, generally, are agreed that rates of fatty-acid liberation are associated with flavor development *(4, 15, 16, 28)*.

It is not surprising, in the light of the fatty-acid data (Table 4), to find that flavor scores and intensity of cheese flavor were not affected by experimental treatments.

Cheese quality. Table 5 presents the body and flavor scores of the cheese at 2, 8, and 36 wk. of age.

TABLE 5

Effect of rennet and calcium chloride on average body and flavor scores of cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese			Raw-milk cheese		
			Lots	Flavor score[a]	Body score[a]	Lots	Flavor score[a]	Body score[a]
	(ml.)	*(%)*	*(No.)*	*(2 weeks)*		*(No.)*	*(2 weeks)*	
A	90	0.00	8	2.75 ± .14	2.59 ± .18	9	3.10 ± .15	2.70 ± .19
B	45	0.00	8	2.65 ± .14	2.71 ± .18	9	2.96 ± .15	2.88 ± .19
C	45	0.02	8	2.66 ± .14	2.72 ± .18	9	3.04 ± .15	3.14 ± .19
				(8 weeks)			*(8 weeks)*	
A	90	0.00	8	2.76 ± .11	2.36 ± .17	9	2.80 ± .29	2.47 ± .21
B	45	0.00	8	2.86 ± .11	2.69 ± .17	9	2.89 ± .29	2.66 ± .21
C	45	0.02	8	2.64 ± .11	2.46 ± .17	9	2.91 ± .29	2.70 ± .21
				(36 weeks)			*(36 weeks)*	
A	90	0.00	8	2.73 ± .28	2.35 ± .17	9	3.17 ± .26	2.56 ± .26
B	45	0.00	8	2.66 ± .28	2.38 ± .17	9	3.05 ± .26	2.66 ± .26
C	45	0.02	8	2.55 ± .28	2.38 ± .17	9	3.24 ± .26	2.66 ± .26

[a] Mean scores and 95% confidence limits. 1—excellent; 2—desirable; 3—satisfactory; 4—objectionable; 5—very objectionable, and 6—unsalable.

The levels of rennet and calcium chloride used had no significant influence either on the flavor score or on the total score of pasteurized- or raw-milk cheese at any age.

The body scores of cheese made by Treatments B and C were slightly inferior at 2 and 8 wk. of age to those of the control cheese, made by Treatment A. These slight differences, however, were not always statistically significant.

Curdiness was the most common body criticism of the young cheese. The extent of curdiness at 2 and 8 wk. is shown (Table 6).

Curdiness in young cheese was exaggerated by using low levels of rennet. The data (Table 6) show this effect clearly. Adding calcium chloride made the effect more pronounced. These trends were still evident, but to a lesser degree,

at 8 wk. of age. Some of the judges classified lack of ideal body at 8 wk. as evidence of persistent curdiness. There was no curdiness in any of the cheese at 36 wk. The greater curdiness at 2 wk. in the cheese made by Treatments B and C could be an important economic factor in the cheese industry, since most cheese is graded when it is from 5 to 14 days old. However, it is extremely unlikely that these differences would be commercially important beyond 8 wk., in cheeses cured like these, at 50° F.

TABLE 6

Effect of rennet and calcium chloride on average degree of curdiness in cheese

Treatment	Rennet per 1,000 lb. milk	$CaCl_2$ added	Pasteurized-milk cheese		Raw-milk cheese	
			Lots	Curdiness[a]	Lots	Curdiness[a]
	(ml.)	*(%)*	*(No.)*	*(2 weeks)*	*(No.)*	*(2 weeks)*
A	90	0.00	8	1.38 ± .27	9	1.78 ± .28
B	45	0.00	8	1.75 ± .27	9	2.36 ± .28
C	45	0.02	8	1.90 ± .27	9	2.50 ± .28
				(8 weeks)		*(8 weeks)*
A	90	0.00	8	0.19 ± .31	9	0.22 ± .34
B	45	0.00	8	0.56 ± .31	9	0.61 ± .34
C	45	0.02	8	0.66 ± .31	9	0.56 ± .34

[a] Means and 95% confidence limits. 0—no curdiness; 1—slightly curdy; 2—distinctly curdy; 3—very curdy, and 4—extremely curdy.

Flavor intensity scores at 2, 8, and 36 wk. showed that a reduction in the amount of rennet, with or without the addition of calcium chloride, had no significant influence on intensity of cheese flavor.

DISCUSSION

The roles played by rennet enzymes and calcium ions in milk-clotting have never been completely defined. However, it has been demonstrated that there are two general phases to the clotting of milk by rennet. An alteration of the casein by rennet enzymes is followed by gel formation, which occurs only in the presence of calcium ions.

Calcium ions by themselves are known to have an aggregating effect on casein micelles *(19, 20, 30)*, and it appears that rennet makes the casein even more sensitive to aggregation by calcium. Therefore, aggregation of casein micelles might be considered as partial clot formation. Thus, the addition of calcium chloride to milk would cause some casein aggregation and require less rennet action to produce a visible clot and a firm curd. Another factor which, no doubt, plays some part in the calcium chloride effect is the influence of this salt in lowering the pH of the milk, thus increasing the curd-shrinking action of the rennet enzymes. These combined effects upon moisture in the curd can actually delay loss of curdiness in normal ripening periods.

Rennet action continues beyond the stage of coagulation and is measurable as percentage of total cheese nitrogen soluble in an acidified sodium citrate solution. When this action is decreased in cheese-making, by using reduced amounts of rennet (Table 4), curdiness persists in the young cheese. Adding calcium chloride and reducing the amount of rennet, establishes two conditions favoring persistence of curdiness.

Curdiness is normally associated with young cheese, but would be criticized at 2 wk. of age if it were excessive, or if the judge expected such curdiness to persist unduly. Curdiness did persist in the cheese made with reduced amounts of rennet and especially in that which contained added calcium chloride.

Hobson and Schaars *(11)* found that the age of mild cheese in grocery stores varied from 4 to 8 wk. At this age, the curdiness found in the experimental cheese made with reduced amounts of rennet, along with added calcium chloride, might still detract from its market value. This same cheese cured for 36 wk. would not be subject to such penalties.

The curing temperature of 50° F. used in these experiments is lower than that commonly used for forced curing of cheese, but higher than the temperatures used for commercial storage. Higher temperatures, if used, would have hastened proteolysis, loss of curdiness, and changes in fat; lower temperatures would have delayed such ripening effects. These facts must be considered in applying the findings of this study to other curing conditions.

This study demonstrated that it was possible to reduce the amount of rennet from 90 to 45 ml. per 1,000 lb. of milk and still obtain satisfactory curd formation in the same time by adding 0.02% calcium chloride to the milk. In making 10,000 lb. of milk into cheese in this way there would be a saving of 15 oz. of rennet, valued at $0.64, based on a price of $5.50 per gallon. It would be necessary to replace that amount of rennet with the equivalent of 2 lb. of purified anhydrous calcium chloride. At $0.24 per pound for a satisfactory grade of calcium chloride, the cost would be $0.48, and the net saving would be only $0.16. If the calcium chloride is purchased in solution as a proprietary preparation, its cost per pound on an anhydrous basis approximates $0.50 at current prices.

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STUDIES RELATED TO THE MANUFACTURE OF SWISS-TYPE CHEESE. I. USE OF HOMOGENIZED MILK

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SUMMARY

Jersey milk standardized to the desired fat:casein ratio with natural or reconstituted skimmilk was used in the manufacture of Swiss-type cheese. All milk was pasteurized at 143° F. for 30 min. In some series, portions of the same milk were homogenized at 0 (control), 500, 1,000, and 2,000 p.s.i. In other series, a blended milk composed of equal parts of 0 and 2,000 p.s.i. treated milk replaced the milk homogenized at 500 or 1,000 p.s.i. Highest yields of cheese were obtained from milk homogenized at 500 p.s.i., followed by blended milk, and milk homogenized at 1,000 p.s.i. Lowest and similar yields were obtained from milk homogenized at 0 and 2,000 p.s.i. A comparison of the quality of the 3-mo.-old cheese showed that only the blended milk cheese excelled the control cheese.

Homogenization of milk was found to lower fat losses in whey as well as to decrease fat leakage in cheese.

Previous workers *(2, 5)* have shown that Swiss-type cheese of legal composition *(3)* can be made from pasteurized milk. Although there exist no published data on the use of homogenized milk in the manufacture of Swiss-type cheese, the work of Matheson *et al. (6)* permits speculation that such may be possible. Homogenization of milk is known to reduce the size of fat globules and has been found to lower fat losses in the whey of Cheddar cheese *(8)*.

This study deals with an evaluation of homogenization as a part of the process for making Swiss-type cheese. For comparative purposes, the cheese was made from pasteurized milk and from pasteurized milk homogenized to various degrees. Adjustment of the fat:casein ratio was accomplished with natural or reconstituted nonfat dry milk.

EXPERIMENTAL METHODS

Jersey milk from the College herd testing 4.8–5.6% fat was secured in quantities sufficient to prepare four 200-lb. lots of standardized processed milk. The raw whole milk in each series was standardized with skimmilk of its kind or reconstituted skimmilk [9 lb. U.S. Estra spray-dried, low-heat nonfat dry milk (N.D.M.) + 91 lb. tap water] to a calculated fat:casein ration of 1:0.8, by using van Slyke's formula *(9)*.

Milk in each series was pasteurized at 143° F. for 30 min. It was cooled in the vat to 110° F., and consecutive portions of 200 lb. each were passed through a Manton-Gaulin homogenizer at given pressures on the way to the surface cooler. All milk was cooled to 40° F. or below, collected in 10-gal. cans, refrigerated overnight, and made into cheese the following morning.

The cheese was made in four identical 50-gal., stainless steel Damrow cheese vats. The same make-schedule was rigidly adhered to in all instances.

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After adjusting the milk to 90° F., 1% active commercial lactic starter was added, followed by the addition of 0.02% $CaCl_2$ dissolved in water. A culture of *Propionobacterium shermanii,* grown in yeast extract for seven days at 85° F., was then added at the rate of 40 ml. per 200 lb. milk. After 60 min. of ripening, the milk was set with commercial rennet at the rate of 90 ml. per 1,000 lb. The curd was cut 30 min. later, using ¼-in. curd knives. Each curd was cut once horizontally and twice each way vertically. Twenty minutes after cutting the curd, one-third of the vat contents was removed in the form of whey. Tap water at 160° F. was added, until the vat contents reached a temperatuer of 120° F.

The curd was agitated and cooked at 102° F. for 60 min. The curd was allowed to settle, pushed to the upper end of the vat, and allowed to mat for 20 min. while draining the whey. The partially matted curd was cut into blocks of suitable size to fill a hoop of the American favorite style. Further treatment of curd and cheese until after salting was identical to that described by Babel and Hammer *(2)*.

The salted cheese was paraffined after the rind was sufficiently dry, and ripened at 70° F. for from 2 to 3 wk. until eyes of desirable size had formed. Further ripening took place at from 50 to 60° F. The cheese was judged when 3 mo. old.

Fat tests on milk, whey, and cheese were made by the Babcock Method *(4)*. Moisture and salt of the 4-wk.-old cheese were determined by an official method *(1)*, whereas the per cent oiling off in cheese was determined as described by Peters *(8)*.

All cheeses were scored by three judges according to a score-card (shown in Table 1), using the criticisms listed by Nelson and Trout *(7)*. The identity of the samples was withheld from the judges in all instances.

TABLE 1

Score-card used in judging Swiss-type cheese

Items considered	Minimum points per item allowed for Swiss-type cheese classified as				Maximum points per item
	Excellent	Good	Fair	Poor	
Flavor	32	29	26	25	40
Holes and appearance	22	19	16	15	25
Texture	18	16	13	12	20
Salt	10	9	8	8	10
Finish	5	4	3	2	5
Total	87	77	66	62	100

RESULTS

Homogenizing milk at increased pressure resulted in decreases in the elasticity of the curd, at cutting time. It also resulted in slower whey expulsion during cooking, reduced color intensity of cheese, and decreases in fat loss in whey. The lower fat losses in whey gave rise to higher per cent fat in the dry matter, as shown in the data (Table 2).

Progressive increases in homogenization pressure decreased the per cent oiling off in cheese. In general, values of from 15 to 20% were judged as most

TABLE 2
Data on composition and yield of Swiss-type cheese

Milk homogenized at p.s.i.	Total lots involved	Average value per item studied: Whey	Cheese			
		Fat	Oiling-off[c]	Fat in T.S.	Moisture	Yield as % of 0 p.s.i.
		(%)				
0	13	0.58	31.5	47.45	39.10	100
500	5	0.32(.50)[b]	19.6 (30.4)	48.84(47.32)	40.01(39.20)	104.4
1,000	8	0.25(.46)	13.5 (31.4)	49.00(47.80)	39.86(39.10)	103.3
2,000	10	0.27(.62)	6.0 (31.4)	49.25(47.30)	38.90(38.92)	100.0
0 + 2,000[a]	5	0.36(.45)	8.7 (32.6)	49.32(48.44)	40.70(39.40)	103.9

[a] 1:1 ratio of 0 p.s.i. to 2,000 p.s.i. milk (referred to as blended milk).
[b] Values obtained on same series with 0 p.s.i. milk.
[c] Based on grams of free solidified fat obtained from 20 g. of ground cheese.

desirable from the standpoint of mouth feel. Whereas cheese made from milk homogenized at 500 or 1,000 p.s.i., or from blended milk, was higher in moisture than was the control cheese, this was not the case with cheese made from milk homogenized at 2,000 p.s.i.

Average yields were the same for cheese made from 0 to 2,000 p.s.i. treated milk. These lots, while similar in per cent moisture, differed in the per cent fat in total solids. Although total solids in whey were not determined, there appeared to be a larger loss of fine curd in whey from milk homogenized at 2,000 p.s.i. than from the control milk. Yields of the other three types of cheese exceeded those of the control cheese. The higher yields were due mainly to higher per cent moisture and higher per cent fat in total solids in cheese.

Data on the quality of 3-mo.-old Swiss-type cheese are presented (Table 3).

TABLE 3
Data on quality of 3-mo.-old Swiss-type cheese

Milk homogenized at p.s.i.	Total lots involved	Average scores per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
0	13	29.2	18.3	16.5	79.0
500	5	29.0(29.8)[b]	18.0(18.7)	16.3(17.0)	78.3(80.5)
1,000	8	28.5(29.0)	17.7(18.0)	16.5(16.3)	77.7(78.3)
2,000	10	28.1(29.2)	16.0(18.3)	14.0(16.5)	73.1(79.0)
0 + 2,000[a]	5	28.8(28.4)	18.3(17.3)	16.3(15.7)	78.4(76.4)

[a] 1:1 ratio of 0 p.s.i. to 2,000 p.s.i. milk (referred to as blended milk).
[b] Scores obtained on same series with 0 p.s.i. milk cheese.
[c] No criticism on salt and finish.

In general, the quality of the cheese decreased with progressive increases in homogenization pressures. Only the blended milk cheese excelled the control cheese in total score, including flavor, holes, appearance, and texture. Specific flavor criticisms associated more frequently with a particular type of cheese were bitter for control cheese, and unnatural for cheese made from milk homogenized at 2,000 p.s.i. The latter type of cheese was criticized also for

such defects as blind, glass, blow holes, and sponge. Criticisms involving all five types of cheese, listed in the order of decreasing frequency, were lacking flavor, picks, small eyes, and bloat. Fewest criticisms were received by cheese made from milk homogenized at 500 p.s.i. and from blended milk; whereas, most criticisms were received by cheese made from milk homogenized at 2,000 p.s.i.

Standardization of milk with either natural or reconstituted skimmilk resulted in cheese of similar composition and quality.

DISCUSSION

The manufacture of Swiss-type cheese from homogenized milk offers some advantages as well as disadvantages. Lower fat losses in whey permit narrowing of the fat:casein ratio, thereby permitting the maufacture of more pounds of cheese per pound of fat. This increases yield. The reduction of fat globules in milk to a smaller, more uniform size decreases the tendency of creaming during setting, thereby resulting in a more uniform distribution of fat throughout the curd and in a cheese with a richer and more mellow flavor. Decreased oiling-off of fat may also be regarded as an advantage, especially if cheese is exposed for any length of time to higher than safe temperatures, be it during manufacture, ripening, merchandising, or in the home.

In contrast to the advantages listed, progressive increases in homogenization pressure reduced the quality of the ripened cheese. Because of the decrease in the elasticity of the curd, it may be that a modification of manufacture and ripening of the cheese would improve the quality without losing any of the advantages listed. One of the more obvious points of attack would be on the ripening of such cheese, particularly the propionic fermentation.

ACKNOWLEDGMENT

The authors are indebted to F. E. Potter for assisting in the judging of the cheese.

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STUDIES RELATED TO THE MANUFACTURE OF SWISS-TYPE CHEESE. II. FACTORS INFLUENCING RIPENING

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SUMMARY

The ripening of Swiss-type cheese, made from standardized pasteurized Jersey milk, passed through the homogenizer at 0 and 2,000 p.s.i., was studied. Varying the amount of propionic culture inocula revealed that in order to obtain cheese of equal quality, a smaller inoculum should be used with milk homogenized at 2,000 than that unhomogenized. The quality of the ripened cheese was not improved by the addition to the milk of KCl or $MnCl_2 \cdot 2H_2O$; the addition of a culture of *Lactobacillus casei* improved it significantly. When cheese made from unhomogenized milk was salted in brine containing KCl, some beneficial effect was observed; this was not the case when milk was homogenized at 2,000 p.s.i.

Pasteurization of milk has been reported to delay the ripening of Swiss cheese *(3, 12)*. Ripening of cheese was enhanced by adding to pasteurized milk, cultures of *Lactobacillus casei*, *p*-amino benzoic acid, and $MnSO_4$ *(3)*. Both Mn^{++} and K^+ have been found beneficial for the growth of *Lactobacillus casei (2, 5)*.

Conditions most favorable for propionic fermentation in Swiss cheese have been reported as temperatures of 72–75° F. *(4)*, pH 5.2–6.8 *(4, 9, 11)*, and an initial population of 100 propionic bacteria per gram of cheese *(4)*. The formation of eyes, or holes, of the desired size, shape, and number is favored by an elastic curd *(4, 9)*, free from mechanical openings and controlled rate of fermentation *(9, 11)*. Since ripening usually results in a more inelastic curd, eye formation should precede the proteolytic breakdown of cheese *(4, 11)*. Excessive propionic fermentation results in cheese with a tough, rubbery body *(7)*, and also prevents or retards the proteolytic breakdown of the curd *(4)*. Free proline *(12)*, calcium and sodium propionate *(1)*, have been reported as being responsible for the desirable sweet flavor in Swiss cheese.

The present study deals with the bacteriological aspects of the ripening of Swiss-type cheese made from pasteurized, and pasteurized, homogenized milk.

EXPERIMENTAL PROCEDURE

Methods other than those described in an earlier paper *(10)* are listed below.

Forty-hour cultures of *L. casei*, grown in peptonized milk broth, were added to milk prior to renneting at the rate of 40 ml. per 200 lb. Potassium chloride and $MnCl_2 \cdot 2H_2O$ were added at the rate of 40 g. and 3 mg., respectively, per 200 lb. of milk. The rate of added KCl to a 23% brine was 200 mg. per liter. Total volatile acid in ripened cheese was determined by the method of Lane and Hammer *(8)*.

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TABLE 1

The influence of amount of propionic culture inoculum upon the quality of 3-mo.-old Swiss-type cheese

Amount of inoculum (ml/200 lb. milk)	Trial	Score per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
20	1[a]	27	20	17	79
40		26	17	13	71
0	2[a]	25	17	15	72
10		26	18	15	74
20		31	21	18	85
40		28	16	13	72
0	3[b]	28	18	17	78
5		28	19	19	81
10		Atypical—discarded			
20		29	19	19	82

[a] Nonhomogenized milk.
[b] Milk homogenized at 2,000 p.s.i.
[c] No criticism on salt and finish.

RESULTS

The influence of amount of propionic culture inoculum upon the quality of 3-mo.-old Swiss-type cheese is shown in the data (Table 1). Inocula of 20 ml. produced cheese superior in flavor, holes, appearance, and texture to cheese containing 40 ml. inoculum, as shown in Trials 1 and 2 with nonhomogenized milk cheese. In Trial 2, inocula of 0 and 10 ml. produced cheese inferior in quality to that containing 20 ml. inoculum. Determination of total volatile acids from cheese of Trial 2 yielded values of 38, 72, 87, and 160 ml. 0.1 *N* acid equivalent per 200 g. of cheese. A similar determination on a lot of 3-mo.-old commercial cheese (not shown), made with 40 ml. inoculum, was found to have a value of 228 ml. of 0.1 *N* acid equivalent. This commercial lot showed excellent eye formation, but was excessive in propionic fermentation and had a tough, rubbery body. Ripening for an additional 7 mo. did not improve the flavor and body of this cheese.

Cheese in Trial 3, made from milk homogenized at 2,000 p.s.i. with 5 to 20 ml. inocula, was similar in quality and superior to the control cheese made without added propionic culture. Cheese made with 10 ml. inoculum was atypical for reasons unknown. Figure 1 shows a photograph of the three lots of cheese from Trial 3.

Swiss-type cheese made from nonhomogenized milk without and with added K^{+} and Mn^{++} resulted in cheese similar in quality, as shown (data in Table 2). Other cheese of the same series, containing added culture of *L. casei* showed superior flavor, holes, appearance, and texture as compared with the control cheese. Cheese made with additions of both salts and *L. casei* was superior to the control cheese, but somewhat inferior to the cheese containing added *L. casei* only. The difference in score between cheese of Trial 1 and Trial 2 was due to the different rate of propionic inoculum used. Cheese in Trial 2 with 40 ml.

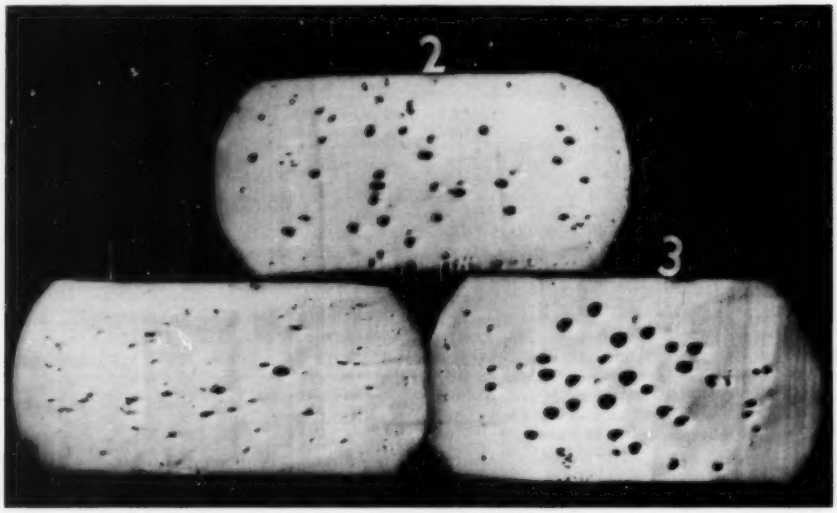

FIG. 1. Swiss-type cheese made from Jersey milk homogenized at 2,000 p.s.i. Amount of propionic culture added to 200 lb. of milk: *(1)* none; *(2)* 5 ml., and *(3)* 20 ml.

inoculum was criticized for bloat and irregular eyes, whereas cheese in Trial 1 was not.

The data (Table 3) show a comparison of nonhomogenized and homogenized milk cheese salted in brine without and with added KCl. The average score for six comparisons of nonhomogenized milk cheese was in favor of brine containing added KCl. Less bitterness resulted when KCl was added, but without it the cheese was judged unclean in flavor. Cheese salted in brine with added KCl

TABLE 2

The influence of additives to milk upon the quality of 3-mo.-old Swiss-type cheese

Types of additive	Trial	Score per item studied			
		Flavor	Holes and appearance	Texture	Total [e]
None (control)	1[a]	27	20	17	79
	2[b]	26	17	13	71
KCl and $MnCl_2 \cdot 2H_2O$[c] (I)	1	26	21	18	80
	2	26	16	12	69
Lactobacillus casei[d] (II)	1	28	22	19	84
	2	30	21	17	83
(I) and (II)	1	28	21	18	82
	2	28	19	15	77

[a] Propionic culture added at the rate of 20 ml/200 lb. milk.
[b] Propionic culture added at the rate of 40 ml/200 lb. milk.
[c] 40 g. KCl + 3 mg. $MnCl_2 \cdot 2H_2O$/200 lb. milk.
[d] 40 ml. peptonized milk broth culture/200 lb. milk.
[e] No criticism on salt and finish.

TABLE 3

The influence of added KCl to brine upon the quality of 3-mo.-old Swiss-type cheese[a] (Average of six lots each)

Treatment of milk	KCl added[b]	Average score per item studied			
		Flavor	Holes and appearance	Texture	Total[c]
Homogenization at 0 p.s.i.	None	27.3	16.5	15.2	74.0
	Added	27.8	18.2	16.2	77.2
Homogenization at 2,000 p.s.i.	None	27.7	16.5	15.0	74.2
	Added	28.3	16.5	14.5	74.3

[a] All cheese made with 40 ml. propionic culture/200 lb. milk.
[b] Added to brine at the rate of 200 mg/liter.
[c] No criticism on salt and finish.

showed slightly superior holes, appearance, and texture. A similar comparison with homogenized milk cheese failed to show any significant difference in the quality of cheese attributable to added KCl.

DISCUSSION

From the data obtained in this study, it is evident that the amount of inoculum of propionic culture plays an important part in the quality of the ripened cheese. It seems advantageous to adjust the inoculum to the treatment received by the milk.

The observed beneficial effect of added *L. casei* in the manufacture of Swiss-type cheese confirms the results obtained with Swiss cheese *(2)*. Failure of K^+ and Mn^{++} to improve the quality of the ripened cheese may be difficult to explain, unless one considers the observations made by Kiuru *(6)* that KCl retards propionic fermentation. Thus, it seems that whereas added KCl is beneficial to lactobacilli in the ripening of Cheddar cheese *(5)*, its presence in Swiss-type cheese is undesirable because of its inhibitory effect upon propionic fermentation.

ACKNOWLEDGMENT

The authors are indebted to F. E. Potter for assisting in the judging of the cheese.

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FREEZING BOVINE SEMEN. III. EFFECT OF FREEZING RATE ON BOVINE SPERMATOZOA FROZEN AND STORED AT —79° C.[1,2]

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SUMMARY

These experiments show the effect of various freezing rates on the decreases in the percentages of motile spermatozoa during freezing and storage at —79° C. Spermatozoa diluted in heated fresh skimmilk showed a progressive decline in motility, irrespective of freezing rate when ampules were removed at —10, —15, —20, —35, —50, and —79° C. Greatest losses in motility with 12 freezing rates occurred between —10 and —35° C. Samples thawed immediately after reaching —79° C. showed the highest motility when a rate of 10° C. per minute from —15 to —50° C. was used. However, following storage for ten days at —79° C. there was a smaller loss of motile spermatozoa with a rate of 5° C. per minute. Very rapid freezing (70° C. per minute) was extremely detrimental; 75% of the total motile spermatozoa present before freezing were lost during freezing and storage for ten days, as compared to only 40% with a freezing rate of 10° C. per minute from —15° C. Similar, but less pronounced, harmful effects were observed when semen diluted in whole milk and yolk-citrate was frozen rapidly. In a storage trial for 0, 2, 4, 8, 16, and 32 wk., the optimum freezing rate for semen diluted in fresh skimmilk or homogenized milk was 1° C. per minute from +5 to —15° C., 5° C. per minute from —15 to —50° C., and rapidly (approximately 20° C. per minute) from —50 to —79° C. The optimum rate for semen diluted in 1:4 egg yolk–citrate was 3° C. per minute from +5 to —15° C. and 5 or 10° C. per minute from —15 to —50° C. The greatest decline in spermatozoan survival occurred during the first 4 wk., regardless of diluents or freezing rates.

British scientists demonstrated that the use of glycerol and slow cooling permitted successful freezing and storage of bull semen at —79°C. *(4, 7, 10, 11).* Using an egg yolk–citrate diluent, Polge *(8)* found that the greatest loss in motility of spermatozoa during freezing occurred at temperatures between —15 and —20° C. However, less damage occurred if the spermatozoa were cooled quickly, over a period of about 2 min., between —15 and —25° C. VanDemark *(12),* also using a yolk-citrate diluent, reported that cooling spermatozoa slowly to —79° C. at a rate of 0.25 or 0.5° C. per minute was inferior to rates of 1 to 4° C. per minute. He also observed *(13)* that cooling at a rate of 2° C. per minute from +5 to —19° C. and 4° C. per minute from —19 to —79° C. resulted in higher spermatozoan survival than cooling at a rate one-fourth as fast, or four times as fast.

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[3] A portion of the data contained in this paper is from a thesis submitted by one of the junior authors (L.A.M.) to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Master of Science.

The literature contains only limited information on the freezing rate required to obtain maximum recovery of bull spermatozoa after freezing and thawing in milk diluents. Erickson *et al. (2)* studied various freezing rates in both egg yolk–citrate and heated homogenized milk diluents, and found that over the crucial range of from −15 to −20° C., 5° C. per minute permitted a higher recovery of motile spermatozoa in yolk-citrate; whereas, a slower rate of 2° C. per minute was better with the milk diluent. Graham and Marion *(3)* also observed that slower freezing rates were required when freezing bull spermatozoa in a whole milk diluent rather than in yolk-citrate. The best rates of motility were obtained when yolk-citrate diluted semen was frozen between 3 and 5° C. per minute and when whole milk diluted semen was frozen between 2 and 3° C. per minute.

This paper reports the results of a series of experiments designed to determine more specifically the optimum rates of freezing bull spermatozoa over the range of from +5 to −79° C. in various diluents, with primary emphasis on heated skimmilk.

EXPERIMENTAL PROCEDURE

Freshly collected semen was diluted so that the initial concentration of spermatozoa was twice the final concentration of 15×10^6 motile spermatozoa per milliliter of diluted semen. Immediately after initial dilution, the samples were cooled to 5° C. over a 3- to 4-hr. period and an equal volume of diluent containing glycerol was added in five equal portions at 6-min. intervals. The final concentrations of glycerol by volume were as follows: heated fresh skimmilk, 13%; heated homogenized milk, 10%; and egg yolk–citrate, 7%. The skimmilk used in these experiments was fortified with condensed skimmilk. In yolk-citrate, the glycerol replaced an equal volume of citrate buffer, and the egg yolk was held constant at 20% by volume. In Experiments 1 and 3, the glycerol fraction of diluter also contained 2.5% fructose (w/v), to provide a final concentration of 1.25% fructose. The final diluted and glycerolated semen contained 1,000 units each of penicillin and streptomycin per milliliter.

Glycerol equilibration time varied with the design of each experiment. In Experiment 1, the equilibration period ranged from 2 to 12 hr.; whereas, in Experiments 2 and 3, a 6-hr. equilibration period was used.

All samples were frozen in 1-gal. Dewar flasks containing one liter of 95% ethyl alcohol. The temperature of the baths was lowered by the manual addition of crushed dry ice. Powdered dry ice was used when more rapid decreases in temperature of the bath were necessary.

The percentages of motile spermatozoa were estimated immediately after the frozen samples were thawed in a water bath at 5° C. To reduce bias, all treatments were coded and the motilities were estimated by a trained assistant who had no knowledge of the particular treatment used with the sample being examined.

In all trials, a Minneapolis Honeywell Automatic Temperature Recorder (Type 153) was used. In Experiment 2, the odd-numbered leads were con-

nected as Thermocouple A and the even numbers as Thermocouple B. Thermocouple A recorded the temperature of the bath, and Thermocouple B was placed in a 10 × 75 mm. test tube containing 1 ml. of diluted semen. With this arrangement, a reading was recorded on a scroll of graph-paper every 30 sec., for the bath and for the semen.

In Experiments 1 and 3, a portable Brown Potentiometer calibrated to read in degrees Centigrade also was used. The M.H. Automatic Temperature Recorder instrument was calibrated against the portable instrument. By connecting all points of the M.H. instrument to one thermocouple, the temperature within the tube of semen was recorded every 15 sec. The portable instrument was used for obtaining the bath temperature.

RESULTS

Experiment 1. It is common practice to freeze semen by gradual cooling to −15° C., followed by more rapid lowering of the temperature after −15° C. This experiment was designed to test the effect of various freezing rates before and after −15° C., upon the motility of spermatozoa during the freezing process and following storage for ten days at −79° C. in heated skimmilk diluent. Ampules were withdrawn for motility estimation from the freezing bath at the following within-ampule temperatures: −10, −15, −20, −35, −50, and −79° C. All semen samples had transformed to the crystalline state before removal, except those withdrawn at −10° C. From +5 to −15° C., freezing

TABLE 1

Effect of various freezing rates before and after −15° C., on the motility of spermatozoa frozen in heated skimmilk diluent

(Mean per cent motility of 13 ejaculates)

Freezing rate per min. from −15 to −50° C.	Freezing rate per min. from +5 to −15° C.	Motility at 5° C. prior to freezing	Motile spermatozoa in ampules removed when ampule temperature reached (°C.)					
			−10	−15	−20	−35	−50	−79
		(%)						
5° C.	1° C.	59	54	51	49	43	43	39
	3° C.	59	56	51	47	44	41	37
	5° C.	59	54	52	49	45	39	36
	Av.	59	55	51	48	44	41	37
10° C.	1° C.	59	54	50	50	45	43	41
	3° C.	59	55	53	52	48	44	42
	5° C.	59	54	51	49	42	42	39
	Av.	59	54	51	50	45	43	41
20° C.	1° C.	59	55	52	51	47	44	37
	3° C.	59	54	54	50	47	44	41
	5° C.	59	52	52	49	46	40	36
	Av.	59	54	53	50	47	43	38
Rapid[a]	1° C.	59	52	51	46	44	42	30
	3° C.	59	54	51	49	47	42	34
	5° C.	59	53	53	48	45	41	30
	Av.	59	53	52	48	45	42	31

[a] Approximately 70° C. per minute.

rates of 1, 3, and 5° C. per minute were used; whereas, from —15 to —50° C. the semen was frozen at rates of 5, 10, and 20° C. per minute, and rapidly. Rapid freezing was obtained by adding finely powdered dry ice to the alcohol bath, and resulted in a freezing rate of approximately 70° C. per minute from —15 to —70° C. With the 5, 10, and 20° C. per minute rates, the temperature of the bath was permitted to drop as rapidly as possible from —50 to —79° C. by the addition of crushed dry ice (approximately 20° C. per minute).

The mean percentages of motile spermatozoa are presented (Table 1). A highly significant interaction was observed ($P<0.01$) between the freezing rates from —15 to —50° C. and the temperatures at which ampules were removed for motility estimations. This interaction resulted primarily from the effect of rapid freezing (70° C. per minute) from —15 to —50° C. on the motility of spermatozoa in ampules withdrawn from the freezing bath at —79° C. Therefore, it appears that the detrimental effect of extremely rapid freezing from —15° C. on spermatozoan motility was not fully exerted until the temperature of the semen was below —50° C.

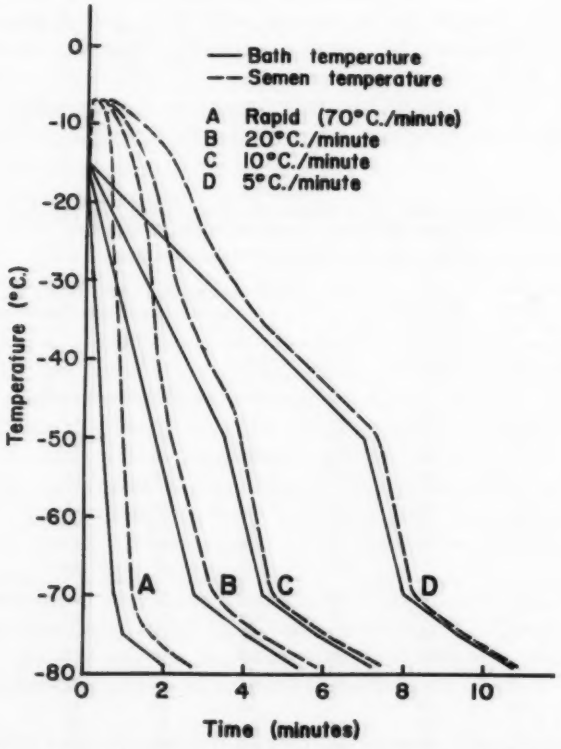

FIG. 1. Effect of lowering freezing bath temperature at four different rates between —15 and —50° C., upon the within-ampule cooling rate of semen diluted in skimmilk.

As shown (Table 1), regardless of freezing rate there was a progressive decrease in spermatozoan motility as the temperature of the semen was lowered. The differences in spermatozoan motility observed in ampules withdrawn from the bath at the various temperatures were highly significant ($P<0.01$). In all cases, a greater decrease in motility occurred between the ampule temperatures of —10 and —20° C. than with any other 10° segment of the freezing curve. The corresponding bath temperatures were —12 and —33° C., using the optimum freezing rate of 3° C. per minute from +5 to —15° C. and 10° C. per minute from —15 to —50° C. The relationship between bath and ampule temperatures for all freezing rates from —15 to —50° C. is shown (Figure 1). With the freezing rate of 1° C. per minute from +5 to —15° C., the temperature of the freezing bath was only 0.5° C. lower than that of the semen within the ampule; whereas, with rates of from 3 to 5° C. per minute, the temperature of the bath was 2° lower than that of the semen. As shown (Figure 2), with the best freezing rate (3° C. per minute from +5 to —15° C. and 10° C. per minute from —15 to —50° C.), 28% of the motile spermatozoa present before

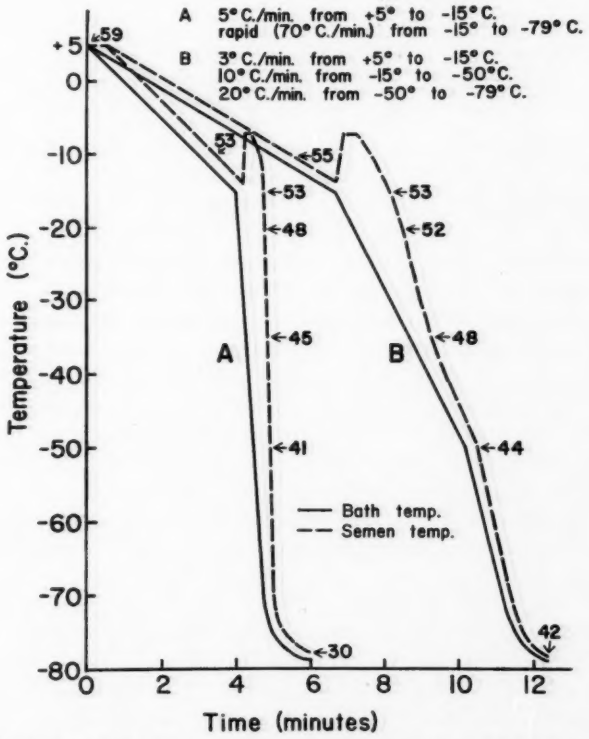

FIG. 2. The decline in the per cent of motile spermatozoa in ampules of semen withdrawn at various temperatures during freezing at two rates.

freezing failed to survive the freezing process. With the poorest freezing rate (5° C. per minute from +5 to −15° C. and rapid from −15 to −50° C.), 49% of the sperm failed to survive the freezing process. Although the highest percentages of motile spermatozoa surviving the freezing process were obtained with a freezing rate of 3° C. per minute from +5 to −15° C. and 10° C. per minute from −15 to −50° C., the differences among the three freezing rates from +5 to −15° C. were not statistically significant.

A separate analyis of variance was conducted on the effect of the 12 freezing rates on the percentages of motile spermatozoa observed immediately after freezing, and those observed in duplicate samples stored for ten days at −79° C. The mean motilities are presented (Table 2). A highly significant interaction

TABLE 2

Effect of various freezing rates on the motility of spermatozoa in heated skimmilk after storage at −79° C. for zero and ten days

(Mean per cent motility of 13 ejaculates)

Freezing rate per min. from −15 to −50° C.[a]	Freezing rate per min. from +5 to −15° C.						Average		Percentage units of difference
	1° C.		3° C.		5° C.				
	(Storage at −79° C.)								
	0 day	*10 days*	*0 day*	*10 days*	*0 day*	*10 days*	*0 day*	*10 days*	
5° C.	39	38	37	36	36	33	37	36	1.0
10° C.	41	35	42	36	39	33	41	35	6.0
20° C.	37	33	41	32	36	28	38	31	7.0
Rapid	30	15	34	16	30	14	31	15	16.0

[a] 20° C. per minute from −50 to −79° C., except for rapid freezing rate, which approximated 70° C. per minute.

between freezing rates from −15 to −50° C. and storage intervals was observed. This is explained by the fact that the slower the freezing rate from −15 to −50° C., the higher were the percentages of motile spermatozoa surviving storage for ten days at −79° C. Although a freezing rate of 10° C. per minute from −15 to −50° C. resulted in a higher percentage of motile spermatozoa immediately after freezing, the slower rate of 5° C. per minute gave slightly higher survival of spermatozoa after storage at −79° C. for ten days. With the extremely rapid rate of 70° C. per minute from −15 to −70° C., an additional 52% of the sperm surviving initial freezing were killed after ten days of storage at −79° C. In this experiment, the rapid rate of freezing from −15° C., and subsequent storage for ten days at −79° C., resulted in a loss of 75% of the motile spermatozoa present before freezing.

Based on the least significant difference between means, a significantly ($P<0.05$) higher percentage of motile spermatozoa after freezing and storage at −79° C. for ten days was obtained with the freezing rate of 3° C. per minute from +5 to −15° C. than at 5° C. per minute. The differences between 1 and 3° C. per minute and 1 and 5° C. per minute were not significant.

Experiment 2. After observing the detrimental effect of rapid freezing from −15 to −79° C., on the motility of spermatozoa diluted in heated skim-

milk, a similar experiment was designed to include heated homogenized milk and 1:4 egg yolk–citrate diluents, as well as heated fresh skimmilk. A uniform freezing rate of 3° C. per minute was used from +5 to −15° C. Freezing rates of 10° C. per minute, 20° C. per minute, and rapid (approximately 70° C. per minute) from −15° C. were employed. The rates of 10 and 20° C. per minute were controlled to −50° C., after which the samples were frozen as rapidly as possible to −79° C. (approximately 20° C. per minute) by the addition of crushed dry ice. Each of ten ejaculates was diluted in each of the three diluents. The mean percentages of motile spermatozoa in the samples thawed immediately after freezing are presented (Table 3).

TABLE 3

Comparison of the motilities of spermatozoa in milk and yolk-citrate diluents when frozen to −79°C. at various rates and thawed immediately

(Mean per cent motility of ten ejaculates)

Freezing rate in °C. per min.	Heated skimmilk	Heated homogenized milk	1:4 Egg yolk–citrate	Average
10° C.[a]	45	50	51	49
20° C.[a]	40	48	50	46
Rapid[b]	28	33	42	34
Av.	38	44	48	

[a] Between −15 and −50° C.; 20° C. per minute after −50° C.
[b] Approximately 70° C. per minute.

Analysis of variance showed a highly significant difference due to freezing rates ($P<0.01$) and a significant difference among diluters ($P<0.05$). Using the least significant difference between means to test differences between diluters, both egg yolk–citrate and heated homogenized milk were significantly ($P<0.05$) better than heated skimmilk.

The interaction between freezing rates and diluters was highly significant ($P<0.01$). In general, ejaculates frozen rapidly in heated milk diluents were lower in motility than portions of the same ejaculates frozen rapidly in yolk-citrate; and samples frozen in homogenized milk were superior to those frozen in skimmilk. However, the rapid freezing rate was detrimental to spermatozoan survival, irrespective of the diluter used.

As shown (Table 3), the mean differences among the motilities of samples frozen at the rates of 10 and 20° C. per minute were small, regardless of the diluter used. However, in all cases the mean motility of samples cooled at 10° C. per minute was higher than of those cooled at 20° C. per minute.

Experiment 3. This experiment was designed to investigate the effect of various freezing rates on the survival of spermatozoa after storage at −79° C. for 32 wk. Each of 16 ejaculates was frozen in heated skimmilk, heated homogenized milk, and 1:4 egg yolk–citrate diluents at the following rates: 1 and 3° C. per minute from +5 to −15° C.; 5 and 10° C. per minute from −15 to −50° C., and approximately 20° C. per minute from −50 to −79° C. Sufficient ampules of diluted semen were prepared to permit storage at −79° C.

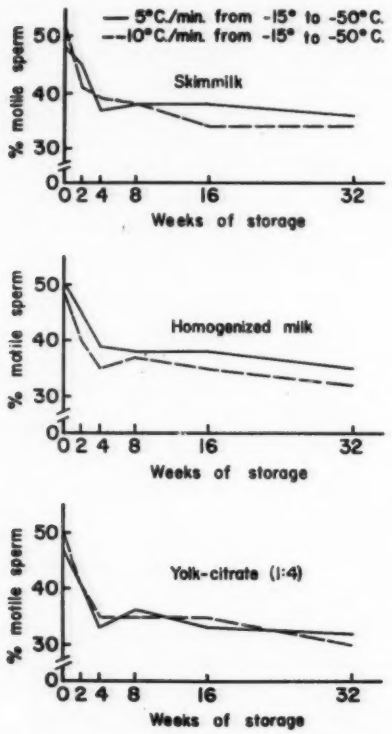

FIG. 2. Effect of two freezing rates from —15 to —50° C., on spermatozoan survival during storage at —79° C. for 32 wk. in heated milk and yolk-citrate diluents.

for intervals of 0, 2, 4, 8, 16, and 32 wk. The final diluted semen contained 1.25% fructose (w/v).

An analysis of variance involving 1,152 motility estimations revealed no significant differences among freezing rates or among diluters. However, a highly significant difference existed among storage intervals. The changes in motility after storage for varying intervals up to 32 wk. are shown (Figure 3). Irrespective of diluents and freezing rates, the major portion of the decrease in spermatozoan motility occurred during the first 4 wk. of storage.

The interaction between freezing rates from +5° to —15° C. and diluents was highly significant; whereas, that between freezing rates from —15 to —50° C. and diluents was significant at the 5% level of probability. Table 4 shows that spermatozoa cooled at 1° C. per minute from +5 to —15° C., and 5° C. per minute from —15 to —50° C., in milk diluents, gave slightly higher survival of spermatozoa after 32 wk. of storage than that observed in samples frozen at the other rates. With yolk-citrate, the faster rate of 3° C. per

TABLE 4

Effect of freezing rates and diluents on the motility of spermatozoa after 32 wk. of storage at −79° C.
(Average of 16 ejaculates and six storage intervals)

Diluent	Freezing rate per minute from +5 to −15° C.	Freezing rate per minute from −15 to −50° C.[a]		Average
		5° C.	10° C.	
Heated skimmilk	1° C.	42	40	41.0
	3° C.	39	39	39.0
	Av.	40.5	39.5	40.0
Heated homogenized milk	1° C.	42	38	40.0
	3° C.	40	38	39.0
	Av.	41.0	38.0	39.5
Egg yolk–citrate (1:4)	1° C.	36	37	36.5
	3° C.	38	39	38.5
	Av.	37.0	38.0	37.5

[a] All frozen 20° C. per minute after −50° C.

minute from +5 to −15° C. and 5 or 10° C. per minute from −15 to −50° C. gave a somewhat higher survival of spermatozoa after 32 wk. of storage at −79° C. Although the differences among diluents were not significant, a slightly higher survival of spermatozoa was obtained in milk diluents than in yolk-citrate diluent, after 32 wk. of storage at −79° C.

The interactions of ejaculates × freezing rates from −15 to −50° C., and ejaculates × diluents were highly significant ($P<0.01$); whereas, those of ejaculates × freezing rates from +5 to −15° C. were significant at the 5% level of probability.

A given set of conditions for freezing bull spermatozoa did not produce optimum recovery of spermatozoa for all bulls, or even for different ejaculates from the same bull. This was shown by the highly significant third-order interaction involving ejaculates, diluents, freezing rates from +5 to −15° C., and freezing rates from −15 to −50° C. In spite of the significant inconsistency from one ejaculate to another, the over-all patterns of interaction (shown Table 4) were significant.

DISCUSSION

These studies show that spermatozoa frozen to −79° C. in heated milk diluents are less resistant to rapid decreases in temperature (70° C. per minute) than spermatozoa frozen in egg yolk–citrate diluent. However, slower freezing rates after −15° C. (5 or 10° C. per minute) gave higher spermatozoan survival in both milk and yolk-citrate diluents. The detrimental effect of rapid freezing after −15° C. on spermatozoan motility in skimmilk diluent was exerted not only during the freezing process but also during subsequent storage at −79° C. for ten days. Of the motile spermatozoa present before freezing, 75% were destroyed after rapid freezing and storage at −79° C. for ten days, as compared to only 40% of those frozen at 10° C. per minute from −15 to −50° C. and stored for ten days.

The results of these experiments clearly show that the term rapid freezing, from —15 to —79° C., as used by various workers, is too general. The actual rate of temperature decline commonly referred to as rapid may vary sufficiently to account for some of the differences in freezability results reported by various workers.

Buch *et al. (1)*, using a yolk-citrate diluent, reported that the greatest decline in per cent of motile spermatozoa occurred during the first 14 days of storage at —79° C. In Experiment 3, the decline extended beyond 14 days and there was a considerable loss in motile spermatozoa between 2 and 4 wk. of storage. The decline between 4 and 32 wk. of storage was very small in both milk and yolk-citrate diluents. Spermatozoa in heated milk diluents retained a slightly higher percentage of motile spermatozoa after 32 wk. of storage at —79° C. than those stored in yolk-citrate diluent.

The results in Experiment 3 showed that a given set of conditions for freezing and storing bull spermatozoa did not give optimum recovery of motile spermatozoa for all bulls, or even for different ejaculates from the same bull. This has been observed by other workers *(1, 6, 9)*.

Based upon the motility of spermatozoa in ampules of semen removed at various temperatures during the freezing process, Experiment 1 showed a progressive decline in motility, with the major portion of the decrease occurring between bath temperatures of approximately —10 and —35° C. This was true of all freezing rates except the 70° C. per minute rate, where a large decrease also occurred between —50 and —79° C. These results agree quite closely with the previous work of Marsh *(5)*, Polge *(8)*, and Lovelock and Polge *(4)*. When spermatozoa were examined immediately after freezing, it was observed that passing through the critical temperature range at 10 or 20° C. per minute, rather than at 5° C. per minute, reduced the loss of motile spermatozoa during the freezing process. However, when samples were examined after ten days of storage at —79° C., it was observed that the faster freezing rates were more detrimental to spermatozoan survival than the slower rate of 5° C. per minute through the critical temperature range.

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INFLUENCE OF SEMINAL PLASMA AND MATURITY OF BOVINE SPERMATOZOA UPON THEIR FREEZABILITY

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SUMMARY

Two experiments were conducted to determine whether the greater postfreezing revival of spermatozoa from the second over the first ejaculate is attributable to differences in seminal plasma or to differences in the cells. In both experiments, spermatozoa were frozen in yolk-citrate diluter. In the first experiment, seminal plasma was interchanged between first and second ejaculates. The superiority of spermatozoa from the second over the first ejaculates remained, whether plasma from the same or the other ejaculate was in the medium or whether the plasma was removed. In the second experiment, the freezability of spermatozoa in the cauda epididymis was superior to that in the ampulla, although not significantly so. The evidence from these two experiments indicates that the superiority in freezability of spermatozoa from the second over the first ejaculate can be attributed to characteristics of the cells and not to differences in seminal plasma.

The preservation of bull spermatozoa by freezing has been successful in many laboratories and artificial breeding organizations and is practiced routinely in some bull studs. About one-third to one-half of spermatozoa are killed during freezing and thawing. Prevention of this wastage may depend upon greater knowledge of the factors associated with survival and death during the freezing process. Available data have suggested that maturity of spermatozoa or changes in seminal plasma might be some of these factors. Two sets of observations from this laboratory *(6)* and a report by Kinney and VanDemark *(11)* indicate that a greater percentage of spermatozoa survives freezing in the second than in the first ejaculate, when they are collected a few minutes apart. Kinney and VanDemark also reported increased survival in successive ejaculates through the fourth, and decreases thereafter. They also observed less resistance to freezing with spermatozoa from the epididymis than with spermatozoa from the more exterior parts of the tract. Elliott *et al. (7)* observed greater freezability of spermatozoa collected after a lapse of from three to seven days from the previous collection than after a lapse of only one day. Progressive improvement in freezability of spermatozoa through four successive ejaculates *(6)* was also noted.

There is some evidence that the chemical composition of spermatozoa in first and second ejaculates differ. Lindahl and Kihlström *(12)* reported a decrease in specific gravity of spermatozoa with successive ejaculates. They interpreted this to mean that dehydration of spermatozoa takes place with increase in maturity. This change in specific gravity has been confirmed by Ohms and Willett *(14)*.

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Furthermore, the composition of seminal plasma changes with successive ejaculates. Changes which have been observed include freezing-point depression *(15)*, pH *(1, 5, 7)*, and phosphomonoesterases *(9)*. Branton *et al. (3)* have shown that sexual excitement can affect chemical composition of seminal plasma, probably by changing the amounts of secretions from the different accessory glands.

These observations raise the question as to whether these or other changes in spermatozoa or plasma or in both are related in some way to freezability. To aid in answering this question, two experiments were carried out and are reported in this paper.

EXPERIMENT I

This experiment was designed to determine if a difference in plasma is responsible for the greater freezability of spermatozoa in the second ejaculate than in the first. Seminal plasma was interchanged between ejaculates or removed from the medium.

Experimental procedure. First and second ejaculates from bulls were collected a few minutes apart. Semen from each ejaculate was placed in five round-bottom test tubes, each containing 5 ml. of yolk-citrate, to give a concentration of 30×10^6 spermatozoa per milliliter. The yolk-citrate diluter consisted of one part yolk and three parts of 2.9% sodium citrate dihydrate and was clarified by centrifuging at 2,500 r.p.m. (about 800 g.) at room temperature for 30 min. This treatment does not reduce the ability of this diluter to maintain livability of spermatozoa during storage at 5° C. *(16)*. Diluted semen in each of the five tubes from each ejaculate received one of the following treatments: *(a)* Uncentrifuged; *(b)* centrifuged twice at 3,100 r.p.m. (2,200 g.) for 3 min. and each time resuspended in supernatant; *(c)* centrifuged as above, removed supernatant, resuspended in yolk-citrate, centrifuged again, removed supernatant, and resuspended in 4.5 ml. yolk-citrate and 0.5 ml. plasma from same ejaculate; *(d)* same as *c*, except that plasma from other ejaculate from the same bull was added after second washing; *(e)* same as *c*, except that 5 ml. of yolk-citrate only was added after second washing.

The seminal plasma added to treatments *c* and *d* had been prepared by centrifuging whole semen at 20,000 r.p.m. (25,000 g.) for 25 min. in a refrigerated Spinco preparative centrifuge, and pipetting off the clear supernatant.

After these treatments, the diluted semen was cooled. Five or 6 hr. after collection, glycerolation was carried out by adding, in five steps, an equal volume of yolk-citrate containing glycerol, at a level of 14% by volume. Then six 1-ml. portions from each test tube were placed in separate, smaller tubes. On the forenoon of the following day, the semen was frozen in an alcohol bath with solid carbon dioxide by cooling from 5 to −12° C. in approximately 14 min., then cooling rapidly with pulverized solid carbon dioxide. Shortly after freezing, the samples were thawed in a water bath at 5° C. with gentle agitation. Motility estimations were then made on the frozen and nonfrozen samples by two people independently and without knowledge of the treatments of the indi-

vidual samples. Estimations were made of spermatozoa from two tubes selected at random from each treatment and were averaged.

Semen from ten collections from seven bulls was employed in this experiment. The volumes of the first and second ejaculates averaged 5.4 and 6.9 ml., and number of spermatozoa per milliliter, 1,785 and 1,529 $\times 10^6$, respectively. Little effort was made to excite the bulls prior to the first ejaculate.

Results. Results are summarized (Table 1). Differences among treatment means within ejaculates are not significant, but the average motility for second

TABLE 1

Progressive motility of spermatozoa from first and second ejaculates, when the spermatozoa were washed, the plasma interchanged or replaced with yolk-citrate, and frozen
(Figures are averages of ten collections)

	Treatment				
	A	B	C	D	E
			Washed		
	Uncentrifuged	Centrifuged, remixed	Same plasma added	Other plasma added	Diluter added
	(%)				
1ST EJACULATE					
Unfrozen	52	51	52	52	50
Frozen	28	27	25	27	24
Revival	54	53	48	52	48
2ND EJACULATE					
Unfrozen	62	63	63	63	61
Frozen	46	41	42	44	44
Revival	74	65	67	70	72

ejaculates is highly significantly greater than for first ejaculates ($P<0.001$). This statement applies whether the diluted semen was or was not frozen. When post-thaw motility means for ejaculates were adjusted by covariance to the same nonfrozen motilities, the difference between ejaculates was still highly significant.

EXPERIMENT II

This study was carried out to determine if spermatozoa from different parts of the bull's reproductive tract differ in their ability to survive freezing.

Experimental procedure. Reproductive organs were dissected from 15 slaughterhouse bulls about 15 min. after they were killed. About 1 hr. later, spermatozoa were flushed from the ampullae, small tubes of the ducti deferentia, and cauda epididymides with yolk-citrate, which consisted of 5% by volume of a 1 *M* solution of sodium citrate, 25% yolk, and 70% distilled water. Spermatozoa were obtained from the caput epididymides by incising the organ in several places and squeezing the spermatozoa into yolk-citrate. Spermatozoa from the different areas were brought to roughly the same concentration in the diluter as judged under the microscope, and were then placed in a refrigerator.

A subsample was removed from each tube and stained with eosin and nigrosin *(2)* on a slide, for later counting of live and dead spermatozoa. Numbers of

proximal and distal protoplasmic droplets were also determined. Approximately 200 spermatozoa were counted in each preparation. Counts were not made of frozen and thawed spermatozoa because of interference by glycerol *(13)*.

The freezing procedure was as follows: About 2.5 hr. after removal of spermatozoa from the organs, the diluter containing the spermatozoa was glycerolated in five steps over a ½-hr. period with the following diluent: 8.6% by volume of a 1 *M* solution of sodium citrate, 25% yolk, 52.4% distilled water, and 14% glycerol. The diluted semen was then frozen as in Experiment I. About 2 hr. later, the samples were thawed and examined.

Results. The data are summarized (Table 2). Motility of nonfrozen spermatozoa from cauda epididymides, small tubes of the ducti deferentia, and ampullae was practically the same. After freezing, however, both progressive and total

TABLE 2

Average motile and live spermatozoa from different parts of the reproductive tract of the bull when frozen and not frozen
(Figures are averages of 15 bulls)

	Epididymides		Small ducts	Ampullae
	Caput	Cauda		
	(%)			
TOTAL MOTILITY				
Unfrozen	2	70	71	70
Frozen	1	41	32	37
Revival	–	59	45	53
PROGRESSIVE MOTILITY				
Unfrozen	0	59	60	60
Frozen	0	29	20	27
Revival	0	49	33	45
LIVE SPERM				
Unfrozen	88	90	85	83

motility of spermatozoa from the small ducts were significantly lower ($P < 0.05$) than those from the cauda epididymides or ampullae. The differences between the latter two were not significant. Practically no spermatozoa from the caput were motile, although they were alive, as indicated by the eosin-nigrosin stain. There were no significant differences in percentages of live spermatozoa among the four locations.

DISCUSSION AND CONCLUSION

In Experiment I, motilities of spermatozoa were similar whether they were in the presence of seminal plasma from the same or the other ejaculate, or in virtual absence of plasma during the freezing process. These results indicate, *(a)* that differences in plasma are not responsible for the superiority in freezability of spermatozoa in the second over those in the first ejaculate, when frozen and thawed, and *(b)* that these differences in ability to survive freezing appear to be characteristic of the cells.

In Experiment II, the spermatozoa from the small tubes of the ducti deferentia had lower motility after freezing and thawing than those from the cauda

epididymides or from the ampullae. Nevertheless, because of the very small number of spermatozoa that can be recovered from the small tubes of the ductus deferens, their reduced motility would not greatly influence the results observed in successive ejaculates. The trend (although not significant) of increased percentages of motile or live spermatozoa obtained at regions progressing from the ampulla to the cauda epididymis is in agreement with the observations made in freezability of spermatozoa from first and second ejaculates.

The number of protoplasmic droplets and their location on the spermatozoa from different parts of the tract, agreed very closely with the data reported by Branton and Salisbury *(4)*.

On the basis of the evidence obtained from these two experiments, it can, therefore, be concluded that the superiority in freezability of spermatozoa from the second over the first ejaculate can be attributed to a factor or factors in the cells and not in the seminal plasma. It is of interest to note that Herman and Swanson *(10)* interchanged plasma from different bulls and observed survival at 5° C. Emmens and Blackshaw *(8)* likewise interchanged seminal plasma among semen samples from different species and then froze them. These different workers concluded that survival of spermatozoa under their respective conditions was more dependent on the characteristics of the spermatozoa themselves than upon the properties of the seminal plasma.

In Experiment I, spermatozoa were exposed to seminal plasma from time of collection to removal by washing, an interval of 0.5 to 1 hr. Changes in spermatozoa related to freezability could be brought about by plasma during this interval. If such is the case, seminal plasma could indirectly be responsible for differences in freezability of spermatozoa in first and second ejaculates, rather than a factor[s] in the cells, as the data in Experiment I suggest. However, the trend in freezability of spermatozoa from different parts of the tract tends to counter this possibility.

The specific changes in spermatozoa that are apparently associated with aging and freezability need yet to be determined.

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ESTIMATION OF SOME VARIANCE COMPONENTS OF BOVINE SEMEN CRITERIA AND THEIR USE IN THE DESIGN OF EXPERIMENTS [1]

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SUMMARY

Semen production records of 68 bulls from five dairy breeds in four studs were used to estimate the bull, ejaculate, breed, season, and interaction variance components of five criteria used to measure semen production. The means and standard deviations of individual observations for the five criteria were: ml. of semen per ejaculate, 8.0 ± 2.4; per cent motile sperm, 66 ± 9.0; billions of sperm per ml. of semen, 1.71 ± 0.62; billions of motile sperm per ejaculate, 8.9 ± 3.9, and billions of total sperm per ejaculate, 13.6 ± 6.0. Component of variance analyses showed that the bull and ejaculate components accounted for more than 90% of the total variance in all criteria except per cent motile sperm. Consequently, these two variances were taken into account in estimating the power of the test by Tang's method. This estimate showed that there was little increase in sensitivity to be gained by taking more than ten ejaculates per bull. It was calculated that to have a 75% chance of detecting a treatment difference of 50% of the mean of motile sperm per ejaculate at the 5% level of significance would require about ten bulls per treatment and five ejaculates per bull.

Although it is common knowledge that the volume, sperm concentration, and per cent of motile sperm of bovine semen ejaculates vary greatly, the authors have found no reports in the literature indicating the size of the components of variance of these criteria for unextended semen. There are in the literature three reports having to do with the estimation and use of variance components of criteria for extended semen *(3, 4, 5)*.

The practical importance of having an estimate of these parameters lies in the fact that with them one may calculate the size of an experiment (number of replications) necessary to detect a specified difference between treatments at a specified level of significance (P_I), and with a specified probability $(1 - P_{II})$ of detecting the difference.

The present report deals with the estimation and use of some of the components of variance contributing to the total variance of ejaculate volume, per cent motile sperm, sperm concentration, motile sperm per ejaculate, and total sperm per ejaculate of dairy sires, criteria having considerable genetic and economic importance in artificial breeding.

MATERIALS AND METHODS

The data available for determining the desired variance components consisted of complete records of sperm production from 68 dairy bulls in four

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[1] The data in this paper are, in part, from a thesis submitted by the senior author to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science, February, 1957.

large bull studs during the period June 1, 1955, through May 29, 1956. These bulls had been ejaculated at intervals ranging from one to 20 days, the modal interval between ejaculates being 1 wk. The per cent of motile sperm in the fresh unextended semen was estimated after dilution in either 2.9% sodium citrate or 0.9% sodium chloride in distilled water. Sperm concentration in the ejaculates was measured with Cenco photelometers,[2] all of which had been calibrated in the authors' laboratory. These procedures for semen examination were essentially those of Willett and Salisbury *(12)*, as modified by Salisbury *et al. (9)*, Willett and Buckner *(11)*, and Bratton *et al. (2)*.

TABLE 1

Measured sources of variation and the expectations of their mean squares

Source of variation		Expectation of mean squares
Season	S	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b} + k_2\sigma^2_s$
Breed	B	$\sigma^2_w + \sigma^2_{e:abs} + k_3\sigma^2_{a:b} + k_4\sigma^2_b$
Interaction	SB	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b} + k_5\sigma^2_{sb}$
Animal (bull)	A:B	$\sigma^2_w + \sigma^2_{e:abs} + k_3\sigma^2_{a:b}$
Interaction	SA:B	$\sigma^2_w + \sigma^2_{e:abs} + k_1\sigma^2_{sa:b}$
Ejaculate	E:ABS	$\sigma^2_w + \sigma^2_{e:abs}$

The extensiveness of the available data permitted selection of bulls and ejaculates within bulls, so as to avoid the excessive computational labor involved in disproportionate subclass number component of variance analysis. The bases for selecting the bulls were: *(a)* Each bull had to have at least one ejaculate of semen in each consecutive 3-wk. period during the 52 wk. of the study and *(b)*, within a stud, each bull had to have a specified minimum number of ejaculates in each of the four 13-wk. seasons of the year. When a bull had more than the specified minimum number of ejaculates for a 13-wk. season, the excess, which was to be discarded, was chosen at random.

The analyses of variance and the component of variance analyses considered the six sources of variation shown (Table 1). The season and breed sources were considered to be fixed and factorial, the animal (bull) source to be random and nested within breed, and the ejaculate source to be random and nested

[2] These were Catalog No. 29330, Central Scientific Co., Chicago, Illinois.

TABLE 2

Number of bulls per breed per stud included in the analyses

Breed	Bull stud I	II	III	IV	Total
Holstein	3	8	7	7	25
Guernsey	2	2	8	5	17
Jersey	3	4	3	0	10
Ayrshire	3	4	0	0	7
Brown Swiss	0	4	0	5	9
Total	11	22	18	17	68

within bull-breed-seasons *(7)*. The following mixed linear model was considered to be appropriate for these data:

$$X_{hijk} = \mu + s_k + b_j + sb_{jk} + a{:}b_{ij} + sa{:}b_{ijk} + e{:}abs_{hijk} + E_{hijk},$$

where X_{hijk} is the value for the h^{th} ejaculate, from the i^{th} animal, in the j^{th} breed and in the k^{th} season. In this model, μ is the mean of the population and E_{hijk} are random errors with a mean of zero and a variance of σ^2_w. All other components of the model were assumed to be uncorrelated variables with means of zero and variances of σ^2_s, σ^2_b, σ^2_{sb}, $\sigma^2_{a:b}$, $\sigma^2_{sa:b}$, and $\sigma^2_{e:abs}$.

The expectations of the mean squares and the coefficients of the components of these expectations (shown in Table 1) were determined by Henderson's procedures *(6, 7)*.

RESULTS AND DISCUSSION

The restrictions applied in selecting the bulls whose semen records were used resulted in the distribution of the bulls among breeds and studs as shown (Table 2). There were seven ejaculates per bull-breed-season subclass for Stud I, ten for Stud II, 11 for Stud III, and 13 for Stud IV, giving a total of 308, 880, 792, and 884 ejaculates per stud, respectively, and a grand total of 2,864 ejaculates.

The averages for the five semen criteria in each stud are shown (Table 3). The standard deviations and the coefficients of variation of the criteria, calculated from the total variance, are shown (Table 4). The similarity among studs of the coefficients of variation within each criterion suggests that these estimates may be reasonably representative of bull studs in general. In fact, they agree closely with estimates for the same criteria recently reported by Bishop and Hancock *(1)* in England.

The analysis of variance for each of the five criteria in each of the four bull studs resulted in the F ratios shown in Table 5. Differences among bulls were highly significant in every test. Although 13 of the 20 tests of seasonal effects

TABLE 3

Average of semen criteria for the four bull studs

Bull stud	Volume of ejaculate	Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate
	(ml.)	*(%)*		*(×10⁹)*	
I	9.0	62	1.82	9.71	15.9
II	8.5	62	1.50	7.95	12.7
III	7.9	72	1.72	9.80	13.6
IV	7.4	65	1.88	8.85	13.7
Av.	8.0	66	1.71	8.93	13.6

were statistically significant, the actual seasonal differences were considered to be too small to be of much practical value in the design of experiments. None of the 20 F tests for differences among breeds was significant, or even approached significance. Much the same situation was true for the season-by-breed interaction.

TABLE 4

Standard deviations and coefficients of variation for all criteria for each bull stud

Bull stud	Volume of ejaculate		Motile sperm		Concentration of sperm per ml.		Motile sperm per ejaculate		Total sperm per ejaculate	
	s	CV	*s*	CV	*s*	CV	*s*	CV	*s*	CV
	(ml.)		*(%)*		*(× 10⁹)*	*(%)*	*(× 10⁹)*	*(%)*	*(× 10⁹)*	*(%)*
I	2.4	41	8.7	14	0.78	43	4.3	44	7.3	46
II	2.9	34	8.5	12	0.57	38	3.9	49	6.0	47
III	2.2	28	12.3	17	0.52	30	4.1	42	5.7	42
IV	2.1	28	6.3	10	0.60	32	3.4	38	5.2	38
Av.	2.4	33	9.0	13	0.62	36	3.9	43	6.0	43

Values obtained for the components of variance are shown (Table 6). Two important findings stand out in this table: First, the ejaculate ($s^2_{e:abs}$) and the bull ($s^2_{a:b}$) estimates of variances are by far the largest components in all criteria except per cent motile sperm. Secondly, many negative values occur in the estimates of the breed (s^2_b) and the season-by-breed (s^2_{sb}) variances. But since negative variances are impossible, these negative values are considered as estimates of parameters which are very close to or equal to zero. The average of the among-stud values for each component, within each criterion, shows that the estimated breed variance (s^2_b)is zero in four of the five criteria, and that the estimated bull ($s^2_{a:b}$) and ejaculate ($s^2_{e:abs}$) variances together account for more than 90% of the total variance in all criteria except per cent motile sperm. Since these two components accounted for most of the variance, they are the only ones considered in the following discussion of the application of variance components in the design of sperm output experiments.

To calculate P_{II} for a two-treatment experiment, Tang's ϕ was computed. For the data in Table 6, the following formula for ϕ was used, as shown by Henderson *(7)*.

$$\phi = \frac{d}{2\,\sigma_{\bar{x}}}$$

In this formula, d is the treatment difference to be detected and $\sigma_{\bar{x}}$ is the standard deviation of a treatment mean. For these data the standard deviation of a treatment mean is estimated from the following formula:

$$s_{\bar{x}} = \sqrt{\frac{s^2_{a:b}}{n_a} + \frac{s^2_{e:abs}}{(n_a)(n_e)}},$$

where $s^2_{a:b}$ is the bull variance, $s^2_{e:abs}$ is the ejaculate variance, n_a is the number of bulls per treatment, and n_e is the number of ejaculates per bull.

Having ϕ, one may find P_{II} from Tang's tables of ϕ shown by Tang *(10)* and by Kempthorne *(8)*. Subtracting P_{II} from one gives the power of the test,

TABLE 5

Summary of F ratios for all studs

Source of variation	Bull stud	Degrees of freedom	Volume of ejaculate	Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate	Significance level	
								5%	1%
S	I	3	3.26	0.37	4.57	9.40	13.96	3.07	4.87
	II	3	5.50	1.97	1.60	0.60	0.94	2.79	4.20
	III	3	3.66	3.55	3.38	5.28	1.62	2.82	4.26
	IV	3	3.54	35.72	9.37	0.72	3.11	2.83	4.29
B	I	3	1.28	0.59	1.03	1.61	0.71	4.35	8.45
	II	4	1.27	0.70	0.78	0.20	0.16	2.96	4.67
	III	2	0.54	0.24	0.98	1.52	1.64	3.68	6.36
	IV	2	0.26	1.95	0.65	0.99	1.08	3.74	6.51
SB	I	9	1.67	0.45	2.09	1.72	2.32	2.37	3.40
	II	12	1.35	0.72	1.11	1.17	1.14	1.95	2.56
	III	6	0.12	0.24	0.43	0.80	0.49	2.31	3.23
	IV	6	1.59	1.60	1.09	2.10	1.88	2.32	3.26
A:B	I	7	14.41	43.92	63.75	32.71	52.92	2.05	2.72
	II	17	27.13	20.99	52.94	73.03	72.84	1.64	1.99
	III	15	46.61	6.03	46.83	25.11	26.98	1.69	2.06
	IV	14	20.69	5.43	26.29	12.52	12.23	1.71	2.10
SA:B	I	21	1.44	1.38	2.81	1.31	0.88	1.60	1.93
	II	51	1.23	2.13	1.65	1.08	1.16	1.37	1.56
	III	45	1.03	6.14	1.41	1.17	0.92	1.40	1.59
	IV	42	1.37	1.61	3.26	2.25	2.22	1.41	1.62

TABLE 6

Estimates of the magnitude of the variance components for the semen criteria for all studs

Component of variance	Bull stud	Volume of ejaculate	% Motile sperm	Concentration of sperm per ml.	Motile sperm per ejaculate	Total sperm per ejaculate
s^2_s	I	0.15	−0.37	0.02	1.00	2.79
	II	0.12	0.32	0.00	−0.01	−0.01
	III	0.03	7.74	0.00	0.26	0.06
	IV	0.05	5.56	0.02	−0.02	0.41
	Av.	0.09	3.31	0.01	0.31	0.82
s^2_b	I	0.19	−7.79	0.00	1.84	−3.80
	II	0.20	−1.31	−0.01	−2.14	−5.39
	III	−0.21	−1.82	0.00	0.54	1.35
	IV	−0.16	0.39	−0.01	0.00	0.07
	Av.	0.00	−2.63	0.00	0.06	−1.94
s^2_{sb}	I	0.18	−1.29	0.03	0.34	1.14
	II	0.05	−0.49	0.00	0.03	0.06
	III	−0.04	−7.43	0.00	−0.04	−0.15
	IV	0.03	0.29	0.00	0.29	0.52
	Av.	0.06	−2.23	0.01	0.16	0.39
$s^2_{a:b}$	I	1.67	50.13	0.36	7.92	35.03
	II	3.06	17.28	0.18	11.04	26.61
	III	2.52	11.18	0.13	5.57	11.39
	IV	1.15	1.87	0.10	1.92	4.23
	Av.	2.10	20.12	0.19	6.61	19.32
$s^2_{sa:b}$	I	0.22	1.75	0.04	0.31	−0.33
	II	0.11	3.89	0.01	0.05	0.24
	III	0.01	45.69	0.00	0.16	−0.13
	IV	0.09	1.03	0.04	0.83	1.83
	Av.	0.11	13.09	0.02	0.34	0.40
$s^2_{c:abs}$	I	3.49	32.69	0.16	6.99	18.89
	II	4.69	34.58	0.14	6.13	14.82
	III	2.43	97.81	0.13	10.16	19.29
	IV	3.04	29.97	0.21	8.65	19.58
	Av.	3.66	48.74	0.16	7.98	18.14
s^2_{total}	I	5.90	75.12	0.61	18.40	53.72
	II	8.23	54.28	0.32	15.10	36.32
	III	4.75	153.16	0.26	16.64	31.80
	IV	4.20	39.12	0.36	11.66	26.66
	Av.	6.02	85.26	0.39	15.46	42.69

that is, the probability of detecting a treatment difference as large as or larger than the one used in the calculation of ϕ.

The power of the analysis of variance F test $(1-P_{II})$ was estimated for some possible two-treatment experiments with varying numbers of bulls per treatment and ejaculates per bull.

Table 7 shows for each criterion in each stud in the study the probabilities $(1-P_{II}$'s) of detecting a treatment difference of 50% of the mean at the 5% significance level, when using two to ten bulls per treatment and five to 50 ejaculates per bull. From these values, it is apparent that in most instances five to ten bulls per treatment and five to ten ejaculates per bull provide relatively good chances of detecting a difference of 50% of the mean. The very high $1-P_{II}$ values for per cent motile sperm reflect the small coefficients of variation of this criterion (shown in Table 4).

Figures 1 and 2 show, for the average stud, the per cent chance of detecting, at the 5% level of significance, a treatment difference of 25 or 50% of the mean number of motile sperm per ejaculate, for varying numbers of bulls per treatment and ejaculates per bull. These figures also show the alternative combinations of numbers of bulls per treatment and ejaculates per bull that have about the same power of the test. For example, in Figure 2, the power of the test for five bulls per treatment and ten ejaculates per bull is approximately 62%, as compared to 69% for ten bulls per treatment and one ejaculate per bull.

TABLE 7

Per cent chance of detecting a treatment difference of 50% of the mean at the 5% level of significance with varying numbers of bulls per treatment and ejaculates per bull

		Number of bulls per treatment											
		2				5				10			
		No. ejac/bull/treatment											
Criteria	Bull stud	5	10	20	50	5	10	20	50	5	10	20	50
Volume of ejaculate	I	28	43	46	46	97	98	98	99	99	99	99	99
	II	24	26	28	28	84	87	89	89	99	99	99	99
	III	26	28	28	28	87	89	92	92	99	99	99	99
	IV	36	41	43	46	96	97	98	99	99	99	99	99
	Av.	28	31	33	33	89	94	94	96	99	99	99	99
Per cent motile sperm	I	59	59	62	66	99	99	99	99	99	99	99	99
	II	86	92	92	92	99	99	99	99	99	99	99	99
	III	86	95	99	99	99	99	99	99	99	99	99	99
	IV	99	99	99	99	99	99	99	99	99	99	99	99
	Av.	82	86	92	92	99	99	99	99	99	99	99	99
Concentration of sperm per ml.	I	14	14	16	16	51	58	58	58	85	85	85	85
	II	18	18	18	18	60	65	65	65	93	93	95	95
	III	24	26	26	28	84	87	89	89	99	99	99	99
	IV	31	33	36	38	92	94	96	97	99	99	99	99
	Av.	20	20	22	22	70	73	77	77	93	93	98	98
Motile sperm per ejaculate	I	16	18	18	18	60	60	65	65	92	93	93	95
	II	11	11	11	11	37	37	37	37	67	67	67	67
	III	18	22	22	22	70	77	80	80	95	97	98	99
	IV	26	31	38	41	89	94	96	97	99	99	99	99
	Av.	16	18	18	18	58	60	65	65	92	93	93	95
Total sperm per ejaculate	I	12	12	12	12	42	46	46	46	76	77	77	77
	II	11	11	12	12	37	42	42	42	67	71	71	71
	III	18	20	22	22	65	73	77	77	95	97	98	99
	IV	28	33	38	43	89	96	97	98	99	99	99	99
	Av.	14	14	16	16	51	58	58	58	85	85	85	85

To increase one's chances of detecting a treatment difference, that is, to increase the power of the test, one has four alternatives. He may *(a)* increase the level of significance, P_I, that is acceptable (e.g., use the 10% rather than the 5% level of significance), *(b)* attempt to detect a larger difference, *(c)* use more bulls or ejaculates per treatment, and/or *(d)* reduce the magnitude of the error

variance. The final choice of power of the test, level of significance, treatment difference to be detected, and number of bulls and ejaculates to be used depends upon the resources available and upon the value of the detectable difference to the experimenter.

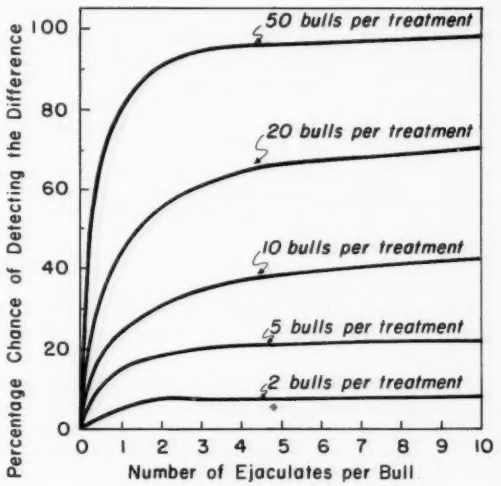

FIG. 1. Per cent chance of detecting a difference of 25% of the mean number of motile sperm per ejaculate, at the 5% significance level, for varying numbers of bulls per treatment and ejaculates per bull.

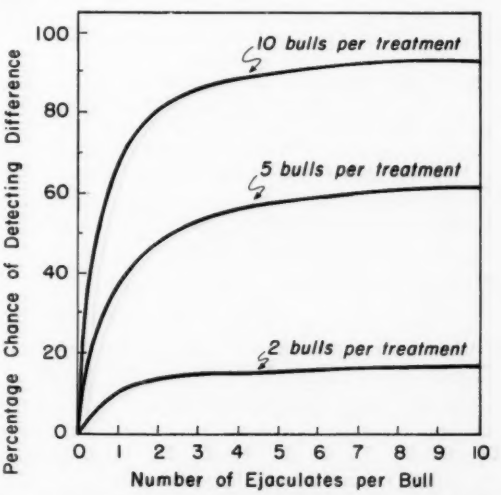

FIG. 2. Per cent chance of detecting a difference of 50% of the mean number of motile sperm per ejaculate, at the 5% significance level, for varying numbers of bulls per treatment and ejaculates per bull.

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EFFECT OF INBREEDING ON PRODUCTION IN HOLSTEINS[1]

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SUMMARY

Data collected over a 25-yr. period from the Iowa State College Holstein herd were used to study the effect of inbreeding on production. The inbreeding ranged from zero to 34% and averaged 7.4%. Records used were standardized to a 305-day-2×-M.E. base. The number of cows used in each analysis varied but, at the most, 534 were available. Intra-sire regressions, using the average of each cow's records, were -1.74 ± 0.57 lb. of butterfat, -54 ± 17 lb. of milk, and $+ 0.003 \pm 0.003\%$ of butterfat for each increase of 1% in inbreeding. There was no evidence of curvilinearity in the effect of inbreeding on fat production. Differences among the individual sires' regressions were not significant. Inbreeding affects production mostly through channels other than reducing general size. This was shown by comparing the regression of production on inbreeding with and without holding heart girth constant. The partial regression was still about three-fourths as large as the simple regression. Partial regression of production on inbreeding, with the dam's production held constant, did not differ appreciably from the regression obtained when the dam's record was disregarded.

In certain situations, inbreeding is an unavoidable consequence of the breeding plan. Among these would be linebreeding programs, and when herds are thought to be of such high genetic merit that breeding animals of higher merit can be found within the herd than in other herds. The primary purpose of the present study was to assess the effect of inbreeding on production, so as to estimate whether the loss (if any) in average phenotypic merit, because of the inbreeding, is likely to exceed the gains which are sought in the plan.

Woodward and Graves *(12, 13)* and Swett *et al. (10)* reported on the effect of inbreeding on production, in the experiment started by the USDA to determine if farmers' herds could be upgraded by using only one registered sire in a sire-daughter mating system. In the first few generations, the inbreeding effects were confounded with breed differences. However, after the cows became predominantly one breed, further inbreeding caused a decrease in milk and butterfat production.

Bartlett and Margolin *(1)* compared 39 outbred and 40 inbred Holstein cows and found that the outbreds were significantly higher in milk and butterfat yield, but the inbreds averaged 0.05% higher in butterfat test. Plum *(8)* found a significant intra-sire correlation of -0.24 between inbreeding and butterfat production in a Jersey herd of 181 cows.

The several experiments which were analyzed by calculating intra-sire regressions of production on inbreeding are summarized (Table 1). The regressions by Tyler *et al. (11)* were calculated with the dam's record held constant.

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TABLE 1

Average changes in production for each increase of one per cent in inbreeding

Authors	Type of record	Cows	B.F.	Milk	Fat
		(No.)	*(lb.)*	*(lb.)*	*(%)*
Davis *et al. (3)*	305-2×-ME	630	−0.66	− 30.8	+0.003
Laben *et al. (4)*	305-2×Jr2	164	−4.88	−209.8	+0.008
Laben and Herman *(5)*	305-2×-ME	299	−2.07	− 66.1	+0.003
Nelson and Lush *(7)*	305-3×-ME	156	−4.5		
Robertson *(9)*	305-2 yr. 10 mo.	80		− 30.0	0.0
Tyler *et al. (11)*	305-2×-ME	89	−2.32	− 73.8	+0.005

SOURCE AND ADJUSTMENT OF DATA

The data came from the Iowa State College Holstein herd from 1930 to June of 1955. After 1930, the herd was almost closed to outside breeding. No outside bulls at all were used after 1934 and no females were introduced after 1937. The purpose of closing the herd was to determine the effects of selection, combined with such inbreeding as would necessarily arise in a finite population of this size. In the selections, production was considered roughly three times as important as type, and information on relatives was given some attention in the selection index. In the earlier years of the experiment, the selection differential for females could not be high because of many troubles, such as pneumonia and reaction to Bang's tests. Sons of the best cows were picked for sires and, as soon as they were of serviceable age, were bred to approximately 40 cows, then were sold with the option of repurchase. This option was exercised only twice.

The 534 cows with records were daughters of 69 sires. Among them they had 1,350 production records averaging 414 lb. of butterfat. The records were standardized to a 305-day, two-time milking, mature equivalent basis.

Most of the inbreeding was slight. The coefficients (Wright's) ranged from 0 to 34% with a mean of 7.4 and a standard deviation of 6.4. The average inbreeding of the cows which calved in 1954 was 11.8%. During the early years of the experiment only a few were inbred at all, and few of these more than 12.5%. In later years, as the background relationship became higher, the average inbreeding increased and the distribution of inbreeding coefficients became less extremely skewed, but the long tail of the curve remained in the direction of high inbreeding, because the bull in service could not have more than one dam and rarely had any full-sisters among his mates.

METHODS AND RESULTS

Simple regressions of production on inbreeding. An inbreeding experiment concerned with dairy cattle is necessarily a long-term project. This is especially so when there is no special effort to increase the inbreeding rapidly. The main reason for this is that the generation interval for dairy cattle is of the order of five years. One cannot hope to hold environmental conditions entirely constant during so long a period as this experiment. Even if it had been physically and

economically feasible to control feeding and management conditions, other extraneous factors, such as temperature, pasture quality, and incidence of disease, are impossible to control wholly. Some important effects of these may even be unknown. Furthermore, the mean genetic composition of the herd can scarcely have remained constant during the past 25 years. The selection for production is likely to have made some genetic changes, natural selection may have been effective in unknown ways, and the very nature of inbreeding permits gene frequency to drift randomly. These sources of change are in addition to any general effects of the inbreeding. The fact that more of the less inbred cows made their records in the earlier part of the experiment, whereas the more highly inbred cows were more frequent in the later years, confounds trends in average inbreeding with time trends in other factors.

Making the analysis on an intra-sire basis would bypass most of the effects of both genetic and environmental time trends, by removing the variance caused by trends and leaving that variance unanalyzed. This is because the comparisons then are only between daughters of the same sire. Since the sires in this herd were nearly always used for only about a year, their daughters were likely to have been nearly contemporary in their lives and production. The pooled intra-sire regression of production on inbreeding is a weighted average of the individual regressions for the daughters of the several sires.

The assumption, usual in the case of regressions, that for complete validity the X's (the independent variable) are measured without error, need not be fulfilled in this case. The X's used in the calculations are inbreeding coefficients calculated according to Wright *(14)*. These are merely expectations of the probable loss in heterozygosis and, therefore, could be in error for any one animal because of the random chance in Mendelian segregation and recombination. However, such an error is merely the amount by which actual heterozygosis is more or less than the computed X. Provided that the only reason for there being any regression at all of production on inbreeding is the inbreeding itself, the true value of Y is correlated with these errors in X. This introduces a term in the numerator of the regression which cancels the effect of the denominator being too large. Berkson *(2)* has advanced a more comprehensive explanation of the reason the regression is unbiased in such cases. Some slight error in the independent variable could arise if differential mortality between the inbreds and noninbreds caused the actually more heterozygous animals to be more likely to live to production age than the less heterozygous ones, which had the same inbreeding coefficients. This would tend to make the regression coefficient a bit nearer to zero than it should be.

TABLE 2

Intra-sire correlations and the regressions of production on one per cent of inbreeding

	B.F.	Milk	Fat
			(%)
Regression	-1.74 ± 0.57	-54 ± 17	$+0.003 \pm 0.003$
Correlation	-0.14 ± 0.05	-0.15 ± 0.05	$+0.05 \pm 0.05$

All of the 534 cows were used in this part of the study. The simple intra-sire regressions and correlations are shown (Table 2). The correlations between inbreeding and production characters would all have been slightly smaller if single records instead of averages had been used.

The present estimates seem low, compared to the estimates Nelson and Lush *(7)* found, using part of these same data. The population would have had to behave quite differently since 1942, because there would certainly be some automatic resemblance in the regressions. To inquire into this, the data were divided into two parts: cows born before 1940 and cows born after 1940. The within-sire regressions of butterfat yield on inbreeding were then calculated for both periods. The regressions calculated for cows born before 1940 were not expected to correspond exactly to the figure from Nelson and Lush. It was not possible to use precisely the same cows and there were some differences in the records used. For instance, their data were on a three-time milking basis. The regression found here for cows born before 1940 was −3.89 lb. of butterfat and for cows born after 1940, −1.33 lb. The difference between the two regressions was not significant; however, it was nearly so ($t = 1.68$ with 462 degrees of freedom). If the regression from Nelson and Lush (−4.5 lb.) is corrected approximately for three-time milking by subtracting 20%, it becomes −3.6 lb.

Effect of inbreeding with dam's record held constant. Morley *(6)* suggested that the simple linear model, such as was used in the previous section, might be inadequate when the population had been subjected to selection. He reasoned that sires would be selected from the genotypically superior female families. The sires, thus being more closely related to the females in these superior familes than to the rest of the herd, would tend to produce their more highly inbred offspring from females above average in transmitting ability. If this bias were important, the simple regression model would underestimate the real effect of inbreeding. The bias will be more when the heritability of the character concerned is high. The extent of this bias will depend on several things, especially on the family structure of the herd and whether the sires are, in fact, much more closely related to the higher-transmitting half of the cows than to the lower-transmitting half. The matings in the Iowa State College herd were made without any conscious attempt either to avoid or to favor inbreeding.

Morley recommended that the phenotype of the dam be considered as an additional independent variable. Then an intra-sire partial regression of production on inbreeding should give an estimate unbiased by any average relation between production of dam and inbreeding of daughter.

Only 484 cows were available for this part of the analysis, because some cows used in the simple regressions had no record available for their dams. This analysis was also on an intra-sire basis.

The correlations between dam's production and daughter's inbreeding are all very small. Only the one for butterfat percentage approaches significance. It is the smallness of these correlations which makes the partial and simple regressions so nearly alike and keeps Morley's argument unimportant in these data.

TABLE 3
Effect of inbreeding with and without considering dam's record

	Regression of daughter's production on each per cent of her inbreeding		Correlation between dam's production and daughter's inbreeding
	Partial	Simple	
Butter yield	-1.72 ± 0.57	-1.72 ± 0.58	-0.001 ± 0.05
Milk yield	-52 ± 17	-50 ± 19	0.05 ± 0.05
Butterfat percentage	0.003 ± 0.002	0.002 ± 0.003	-0.09 ± 0.05

Effect of inbreeding with heart girth held constant. According to Nelson and Lush *(7)*, increased inbreeding caused a decrease in the cow's size in the Iowa State herd. They found that size was affected most at 2 yr. of age and that the effect decreased as the cows got older. At 5 yr. of age, the regression of size on inbreeding was positive. They postulated that inbreeding slowed down the growth rate but did not limit utimate mature size. However, their data at the older ages were few. In the present analyses, it was thought desirable to find whether the depressing effect of inbreeding on production was due to decreased size of the more highly inbred cows, or if inbreeding was acting on production through channels other than its effects on size.

First records were used for this part of the analysis. When the cows started their first production records, their ages ranged from 21 to 36 mo. The records were converted to maturity, in order to eliminate differences caused by this range in ages. The cows were measured at as nearly 2 yr. of age as was practical. Seldom did the cow's age at measurement deviate more than 1 wk. from its second birthday. Measuring the cows at a fixed age, while they naturally varied in their age at first calving, allowed differences in the stage of gestation to introduce some extraneous variation in the measurements. Heart girth was used because it was thought to be the best measure of general size (among those measurements available) and was not likely to be influenced much by stage of gestation.

Multiple regression (intra-sire) was the method of analysis, just as in studying the dam's production. The 495 cows which also had a 2-yr.-old heart girth measurements were the progeny of 54 different sires. This and subsequent analyses were confined to butterfat production, because inbreeding seemed to have little or no effect on butterfat percentage.

The simple regression of production on inbreeding was -2.72 ± 0.63 lb. of butterfat; whereas, the partial regression of production on inbreeding with heart girth held constant was -2.05 ± 0.64 lb. The simple regression of heart girth on inbreeding was -0.30 ± 0.05 cm. for each increase of 1% in inbreeding. The simple correlation between heart girth and butterfat production was $+0.22 \pm 0.05$. All of these regressions and correlation coefficients are significantly different from zero at a probability level of less than 1%. The difference between 2.72 and 2.05 seems to indicate that the depressing effect of inbreeding on production is in part a consequence of its having depressed size or growth rate,

but the 2.05 indicates that much of its effect on production goes through one or more other channels.

Test for linearity and homogeneity of regressions. It seemed desirable to investigate whether the regression of production on inbreeding is, in fact, linear. If all the effects of the genes were simply additive, there is no apparent reason for inbreeding per se to have any effect on production. If all of the nonlinear effects of the genes are those due to some degree of dominance of genes for high production (even including overdominance), inbreeding would cause production to decrease, but the decrease should be linear with the inbreeding coefficient. Certain kinds of epistatic effects would cause a curvilinear decrease in production. For instance, those epistatic effects which involve the simultaneous presence of the heterozygote dominance deviations in at least two different pairs of genes would cause a curvilinear decrease. The more pairs of genes involved in such combinations, the faster inbreeding would break up the combinations. If inbreeding reached a level of 50%, theoretically only 1/16th of the 4-pair, one-eighth of the 3-pair, one-fourth of the 2-pair, and one-half of the 1-pair combinations would be left.

The regressions presented in the previous sections were average regressions for all sires. It seemed appropriate to find whether the regression coefficients of individual sires differed from each other more than would be expected by chance. Such differences might be expected if the inbreeding decline were due only to the uncovering of recessives and the sires varied much in the number of these they carried. To test these two things, the analysis of variance (shown Table 4) was calculated. In order to get a reasonable number of cows within

TABLE 4

Analysis of variance to test for linearity of the regressions and for homogeneity of the regressions of individual sires

Variation due to:	Sum of squares	Degrees of freedom	Mean square
Total	2,172,262	501	
Among sires	419,599	44	
Within sires	1,752,663	457	
Among F_x classes within sires	503,576	157	3,207
Pooled intra-sire regression	45,951	1	45,951**
Among regressions of individual sires	91,608	44	2,082
Deviations from linear regressions	366,017	112	3,268
Within F_x classes within sires	1,249,087	300	4,164

** Statistically significant beyond the .01 level.

each inbreeding class, the cows were groped in intervals of 3% of inbreeding. Sires with only one or two daughters were discarded, because they did not contribute to the analysis. This left 502 cows by 45 sires. Again, the average of all of a cow's lactations was used as a measure of its butterfat production.

The surprisingly small mean square for differences among the regressions of individual sires seems to indicate that the decline in production caused by inbreeding is due to many genes, each of which would necessarily have a rather small effect. If this decline were due to inbreeding uncovering a very few major recessives with important detrimental effects, surely some of the sires would

have been heterozygous for more of these major genes than others. Thus, differences among sires should have been found. The smallness of this mean square also appears to indicate that the variation among sires in transmitting ability was small. A positive correlation between the inbreeding of a sire's daughters and their relationship to him is a necessity. Then, if a sire had an individual transmitting ability far above the level of the herd, his inbred daughters, being more closely related to him, should for that reason have higher production. Conversely, if some sires with transmitting ability far below average were picked by mistake, their more highly inbred daughters should be farther below the herd average.

The small mean square (3268) in Table 4 for deviations from linearity for each individual sire's regression line indicates that the true regressions are linear. Otherwise, this mean square would be expected to be larger than the mean square just below it. The data actually agree a little better than expected with the hypotheses that the regressions for individual sires are *(a)* equal and *(b)* linear.

DISCUSSION

The level of inbreeding reached in this herd, after more than 20 yr. essentially without introduction of outside blood, emphasizes that a herd of this size can be closed for a long time without the inbreeding reaching a high level. Actually, since production has been increased above starting levels, the accumulated inbreeding might be considered as an additional benefit, since that gives some promise that the cows would transmit their high level somewhat more uniformly. If the number of sires in use at any one time had been still larger, and an effort had been made to avoid mating highly related animals, the level of inbreeding could have been kept even lower.

Intra-sire regressions of −1.74 lb. of butterfat, −54 lb. of milk, and +0.003% butterfat, for each increase of 1% of inbreeding, are well within the range of the findings reported by other workers. Most of the investigations, including this one, have found a slight positive regression of butterfat per cent on inbreeding. It is well known that environmental conditions which affect milk and butterfat production adversely tend to raise butterfat percentage. Perhaps this is enough to explain the apparent slight effect of inbreeding on butterfat percentage.

Although not quite significant, the difference between the regressions for cows born before and after 1940 seems unduly large. There is approximately only one chance in ten that a difference this large would have been found, if, in fact, there were no true difference. Selection has been much stronger since 1940 than before, and it seems plausible that this might have had some influence on the effect of inbreeding. If the regression were curvilinear, there could be a difference in the two linear regressions, because the more highly inbred cows tended to make records after 1940. However, no evidence was found to indicate that the regression was not linear.

Holding the dam's record constant did not alter the regression coefficients appreciably. This happened because the correlations between the dam's pro-

duction and daughter's inbreeding were small and varied in signs. Considering that approximately one-tenth of the data had to be discarded, and that the simple and partial regressions were nearly alike, holding the dam's record constant seems not to have added enough information to be worth while in these data.

Reduction of the simple regression of -2.72 lb. to -2.05 lb. for the partial regression of inbreeding on first lactation fat production, when heart girth is held constant, indicates that inbreeding causes some of the decrease in production through its detrimental effect on body size. This would be expected from the a priori knowledge of the positive correlation between size and production and the negative correlation between inbreeding and size. Although this reduction is only of the order of one-fourth, perhaps it should be considered when assessing the effect of inbreeding on production, because the smaller cow is not necessarily less economical even though on the average it produces less.

A difference as large as the one between the regression of -2.72 for first records and -1.74 for average records seems too large to be written off as likely to have been caused by chance alone. A plausible explanation of the differences between the regressions for first and average records is that the depressing effect of inbreeding on size and production is materially reduced at older ages; i.e., that inbreeding acts more to lower the rate of development than it does to limit eventual size and production at maturity. Therefore, when average records are used some, but not all, of the adverse effect of inbreeding on production would be removed.

It would be interesting to know whether the effect of inbreeding on production is reduced or eliminated at maturity. Unfortunately, when the population has been subjected to selection, no way to evaluate the effect of inbreeding at different ages is apparent. If the cows are selected on their own performance (as was the main basis for their selection here), the older cows would tend to be the better producers, regardless of their inbreeding coefficients.

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PERMANENCE OF TYPE RATINGS IN YOUNG HOLSTEIN HEIFERS[1,2]

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SUMMARY

To determine the repeatability of type ratings in young heifers, 544 animals in five herds were evaluated at 3, 6, and 12 mo. of age. Evaluations were made on a ten-division score-card basis, and each animal was scored independently by two evaluators working simultaneously. Repeatabilities of the various type components were estimated by intra-heifer, intra-herd correlations. The correlation values between 3- and 6-mo. scores ranged from 0.2 to 0.3 for the different score-card divisions. Values between 6 and 12 mo. showed a somewhat wider range, and those between 3 and 12 mo. were somewhat lower. Variations among herds and among score-card divisions within herds are a definite limitation upon the usefulness of this information in small groups of heifers. Definite, characteristic age changes were observed in all score-card divisions. The most consistent of these were a progressive improvement in shoulders and chest (more marked between 3 and 6 mo., but also significant between 6 and 12 mo.), a drop in scores for rump and thigh between 6 and 12 mo., and a marked improvement in feet and legs between 3 and 6 mo. With some exceptions, there was a rather strong tendency for heifers to be scored higher at 6 mo. of age than at other ages. This was particularly noticeable in total score.

Since type classification has been in general use, the permanence of such ratings has been of considerable interest. Most of this interest has been in cows of producing age. The possibility of phenotypic and genetic correlations between type and production has also been investigated. Although type in heifers has received some attention, no study has been reported in which the heifers were evaluated at specific ages. In most studies, type has been evaluated only in rather broad, general terms, most of the interest being in a single, final rating.

The present study was designed to evaluate the changes in body conformation of the young heifer, by scoring type in considerable detail at specific ages. Some of the changes which occur between 3 and 12 mo. of age are discussed.

Johnson and Lush *(7)* found the intra-cow correlation between different classification ratings to be 0.55 when the ratings were made by a three-man committee, and 0.34 when ratings were made by individual judges. Hyatt and Tyler *(5)* found the intra-cow, inter-judge correlation between different ratings in one Ayrshire herd to be 0.55. Benson *et al. (1)* estimated the repeatabilities of the ten divisions of the Ayrshire score-card to range from 0.3 to 0.6 for cows. Similar repeatabilities for heifers ranged from 0.0 to 0.3. Hyatt *et al. (6)* found the regression of average cow rating upon average heifer rating to be 0.45.

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[2] These data were taken from material submitted by the senior author in partial fulfillment of the requirements for the Doctor of Philosophy degree, The Ohio State University, Columbus, Ohio.

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Harvey *et al. (4)* concluded that type changes in heifers between 6 and 24 mo. were largely unpredictable.

METHODS

Five hundred and forty-four Holstein heifers in the Ohio NC-2 Breeding Project were evaluated for type at 3, 6, and 12 mo. of age. This group included all of the heifers raised through these ages during a three-year period ending February, 1954, in five herds owned by the Ohio Department of Mental Hygiene and Correction. These herds, and the number of heifers in each, are: Athens State Hospital, 72; Dayton State Hospital, 107; London Prison Farm, 158; Orient State School, 73, and Toledo State Hospital, 134. These herds are visited bimonthly on approximately the same date each time, and the heifers are evaluated at the time of the visit nearest the selected ages. With a few exceptions, the evaluations used are the averages of the scores of two judges working independently at the same time. Four different judges did appreciable amounts of the work on the Toledo herd, but most of the evaluations in the four other herds were made by the same two men. An Ayrshire score-card was used, slightly modified. The judges worked together in several practice sessions, to standardize their interpretations of the various score-card divisions. The designations of the last two divisions were changed from general quality and breed character to dairy character and general appearance, because these terms seemed to be more descriptive. Other divisions may not have been defined exactly as they were originally intended. The final, or total, score is the sum of the ten division scores. Special emphasis was placed upon rating each score-card division as objectively as possible, without reference to any preconceived idea of over-all rating or total score.

These data were analyzed for repeatability and age differences.

RESULTS AND DISCUSSION

Since herd differences in average score were apparent, repeatability was first estimated within herds. These five estimates were then combined by converting each r to weighted z, Snedecor *(9)*. The herd differences in repeabability were tested for significance, and an average r was computed for each division in which the herd difference was not significant at the 1% level of probability. These results are summarized (Tables 1, 2, and 3). In the 3 to 6 mo. comparison, the difference in repeatability was obviously due to the low r in the London herd. In the two other age comparisons (6-12 mo. and 3-12 mo.), significant differences were found among herds in the repeatability of scores for size and placement of teats. These differences could not be attached to particular herds. Finally, the data from all herds were pooled, to give a single estimate for each score-card division. In general, this latter method yields estimates which are equal to or higher than those derived from the average z's. The lower r's for udder size and shape in both the 3-6 and 3-12 mo. comparisons are notable exceptions. In this division, different herds appear to have conflicting trends between three mo.

TABLE 1

Repeatability of type evaluations from 3 to 6 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.379	0.303	0.268	0.570	0.385	0.460	0.351	0.216	0.551	0.470	0.475
Dayton	107	0.301	0.411	0.319	0.256	0.441	0.183	0.080	0.289	0.364	0.350	0.466
London	158	0.214	0.101	0.301	0.154	0.272	0.300	0.342	0.446	0.146	0.144	0.079
Orient	73	0.170	0.341	0.316	0.394	0.364	0.174	0.091	0.453	0.268	0.248	0.357
Toledo	134	0.280	0.280	0.292	0.342	0.235	0.174	0.184	0.127	0.312	0.350	0.321
All	544	0.313	0.393	0.320	0.335	0.468	0.143	0.242	0.282	0.333	0.378	0.445
Average		0.263	0.267	0.301	0.313[a]	0.325	0.253	0.223	0.317[a]	0.305[a]	0.296	[b]

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 2

Repeatability of type evaluations from 6 to 12 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.370	0.217	0.181	0.466	0.307	0.248	0.328	0.345	0.359	0.113	0.326
Dayton	107	0.393	0.376	0.032	0.390	0.426	0.309	0.301	0.261	0.125	0.186	0.410
London	158	0.187	0.206	0.221	0.378	0.187	0.144	0.258	0.484	0.279	0.222	0.281
Orient	73	0.211	0.309	0.317	0.368	0.147	0.009	0.163	0.307	0.014	0.255	0.289
Toledo	134	0.255	0.303	0.256	0.297	0.054	0.144	0.134	0.025	0.331	0.342	0.110
All	544	0.295	0.378	0.260	0.410	0.350	0.466	0.466	0.445	0.274	0.427	0.516
Average		0.272	0.308	0.201	0.372	0.216[a]	0.174	0.234	[b]	0.240	0.239	0.273

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 3

Repeatability of type evaluations from 3 to 12 mo. of age

Herd	No.	Head and neck	Shoulders and chest	Middle and loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
Athens	72	0.032	0.366	0.054	0.345	0.209	0.167	0.336	0.058	0.407	0.149	0.234
Dayton	107	0.176	0.171	0.275	0.158	0.246	0.024	−0.017	0.081	0.190	0.006	0.202
London	158	0.178	0.012	0.072	0.202	0.156	0.318	0.267	0.423	0.231	0.169	0.207
Orient	73	0.248	0.361	0.388	0.366	0.293	0.283	0.293	0.346	0.274	0.117	0.395
Toledo	134	0.130	0.140	0.241	0.049	−0.077	0.028	0.014	0.043	0.129	0.279	0.039
All	544	0.201	0.266	0.222	0.216	0.292	0.102	0.205	0.203	0.277	0.245	0.327
Average		0.157	0.171[a]	0.197	0.198	0.180	0.167[a]	0.171[a]	[b]	0.228	0.157	0.196

[a] Difference between herds significant at the 5% level.
[b] Difference between herds significant at the 1% level.

TABLE 4

Effect of age on type ratings of heifers

	Mean scores										
Age (mo.)	Head and neck	Shoulders and chest	Middle loin	Rump and thighs	Feet and legs	Shape and size	Udder attachment	Teats	Dairy character	General appearance	Total
3	81.6a	80.4a	82.4a	81.6a	79.6a	81.2a	81.2a	81.5a	82.1a	81.4a	812.8a
6	81.6a	81.4b	83.6a	81.8a	80.4b	82.1b	81.9b	82.1b	82.7b	82.2b	819.8b
12	80.8b	81.8c	82.9b	79.9b	80.6b	81.5c	81.6b	81.4a	82.2a	81.2a	813.9a
	Analysis of variance										
Source	Mean square										
Ages	116[b]	298[b]	191[b]	614[b]	159[b]	122[b]	146[b]	588[b]	66.5[b]	168[b]	7715
Herds	139[b]	336[b]	98.0[b]	124[b]	624[b]	476[b]	577[b]	40.8[b]	88.5[b]	544[b]	23985
a × h	11.4[a]	14.9[b]	9.75	16.5[b]	9.12	144[b]	67.9[b]	51.1[b]	5.09	24.4[b]	1150.5
Error	4.58	5.27	5.64	5.57	7.72	4.86	5.48	4.45	3.66	5.49	160.54

a, b, c—Age means in each score-card division which are followed by the same letter are not significantly different; means followed by different letters are significantly different. The methods described by Duncan *(3)* were used to test these differences.

[a] Difference between herds significant at the 5% level.

[b] Difference between herds significant at the 1% level.

and the other ages. In some instances, the combined data probably yield a more generally applicable estimate of repeatability. However, any environmental influences which affect type scores contribute toward higher estimates, and this fact should be recognized. The estimates derived from the average z's appear to be more conservative.

Age differences in the data were tested for significance by analysis of variance. These results are summarized (Table 4). The differences between ages and differences between herds were significant well beyond the 1% level of probability in each score-card division. This was not true, however, for all divisions for age × herd interaction. The apparent disagreement with the findings of Harvey *et al. (4)* is not considered serious, in view of the difference in age of heifers studied, the age-grouping procedure of those workers, compared with the exact ages (± about seven days) in the present study, and the much more detailed score-card used here, in contrast to the more general score-card divisions used by the Idaho workers.

The most consistent age changes were found in shoulders and chest, rump and thighs, and feet and legs. Scores for shoulders and chest tend to improve progressively from 3 to 12 mo. of age, but the greatest improvement is made before the animals are 6 mo. of age. Each herd exhibits the same trend, except the Toledo herd, which shows no improvement between 6 and 12 mo. A possible explanation of this improvement is suggested in unpublished data by Clifton and Rickard *(2)*, which show that the spinal processes usually grow above the top of the shoulder blades during this age period. Of 256 heifers in three herds, only ten had spinal processes above the shoulder blades at 3 mo. of age. The processes began to protrude between 3 and 6 mo. on 125 heifers, between 6 and 12 mo. on 76, and had not protruded at 12 mo. on 27 heifers. The remaining 18 heifers showed inconsistent changes. The protrusion of these processes, no doubt, make the withers appear sharper and the shoulders more neatly arranged.

Scores for rump and thighs drop markedly between 6 and 12 mo. This is in apparent agreement with the findings of Leighton and Graves *(8)*, who found that rump slope increased with age.

Feet and legs show significant improvement between 3 and 6 mo., but the Dayton herd shows marked improvement between 6 and 12 mo. Since the heifers in this herd are somewhat retarded in growth, an environmental effect is suggested.

Other age changes which appear to be consistent in most herds are a drop in head and neck scores between 6 and 12 mo.; an increase in scores for middle and loin between 3 and 6 mo., followed by a downward trend at 12 mo., and a general tendency for heifers to score higher at 6 mo. in all udder divisions, dairy character, general appearance, and total score. Herd differences, particularly in the last two, seem to reflect environmental differences, and are greatest at 12 mo. of age. Herd differences were also quite apparent in the scores of the udder divisions. Although environment may account for some of the herd differences, it does not seem to furnish adequate explanations for all differences. The erratic repeatability estimates in these divisions can be explained by these herd differ-

ences in age trends. In addition to herd differences, certain heifers exhibit marked departure from the general trend. The possibility that certain related groups within herds may exhibit their own characteristic trends is suggested.

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BACTERIOLOGICAL STUDIES OF UTERI OF CLINICALLY NORMAL COWS OF LOW FERTILITY[1]

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SUMMARY

This study was undertaken to determine if the uteri of clinically normal repeat-breeder cows contained microorganisms at 16 days postbreeding, a stage immediately followed by a high incidence of embryonic death. Sixty-nine repeat-breeders were slaughtered, the uteri removed aseptically, and flushed with 15 ml. of 0.1% tryptone broth. Various media were inoculated with the recovered fluid and incubated in air, in 15% CO_2 and reduced air, and in an anaerobic jar. Triturated endometrial tissue of 35 animals in the study was similarly cultured. Microorganisms were recovered from the uteri of eight (11.6%) of the 69 repeat-breeders. A yeast, *Candida krusei*, was isolated from four uteri, an *Escherichia* species from one, a *Pseudomonas* from one, *Brucella abortus* from one, and a paracolon from one. Embryos were recovered from 31 of 62 uteri from cows with anatomically normal reproductive tracts; three embryos were recovered from the eight infected uteri. Vaginal mucus samples were obtained from 35 of the animals at from 11 to 13 days postbreeding and at slaughter. One cow had a positive mucus agglutination test for *Vibro fetus*. Serum agglutination-lysis tests for leptospirosis were made on all animals. None was positive. Microbial infection apparently accounted for few of the embryonic deaths in these clinically normal repeat-breeders.

Embryonic death is a major cause of infertile services in dairy cows of low fertility; this is especially true in those animals that show no recognized clinical manifestations for which known therapy is indicated *(2, 5, 13, 14)*. It appears that the majority of these deaths in such repeat-breeding cows occurs shortly after the 16th day of gestation *(5)*. The presence of low-grade infections, for which characteristic pathology is not detected, has frequently been proposed as the cause of early embryonic mortality in these cows. Infusion of the uteri with antibiotics at the time of, or immediately prior to, breeding, however, did not lessen the embryonic death *(15)*. But the apparent time of death of the embryo in these cows (16 + days) might be the result of infectious agents entering the uterus after the time of treatment. Contradicting the work of Ulberg *et al. (15)*, Lindley *(9)* has reported considerable increase in the conception rate of repeat-

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breeding cows, with apparently low-grade infections, by intra-uterine infusion of antibiotics and sulfonamide solutions one or two days following service.

The possible role of microorganisms in the repeat-breeding problem has received considerable attention and has been reviewed extensively *(1, 10)*. More recently, much attention *(3, 11)* has been paid to *Vibrio fetus* as a cause of lowered fertility in the repeat-breeding dairy cow. In a recent extensive study, Gunter *et al. (4)* demonstrated the presence of pathogenic micrococci, diphtheroids, and streptococci from the uteri of repeat-breeding cows; whereas, from normal-breeding animals predominantly saprophytic strains of bacteria were obtained. Furthermore, Gunter *et al. (4)* report that 38% of the samples from the uteri of normal cows was sterile; whereas, only 5% of similar samples from repeat-breeders was sterile.

Our present study was undertaken to determine the percentage of repeat-breeder cow uteri containing microorganisms at 16 days postbreeding, an estimate thus being obtained of the importance of infectious agents in the uterus as causes of the embryonic death occurring shortly after 16 days. The assumption was made that microorganisms causing subsequent embryonic death would likely be present in the uterus at the time of slaughter.

MATERIALS AND METHODS

Selection and handling of cows. Sixty-nine cows of low fertility were used in the study. They were obtained between April, 1955, and March, 1956, from farms throughout Wisconsin and east-central Minnesota and were selected on the following bases: *(a)* Bred at least four times without apparent conception, *(b)* bred last within the past 90 days and returned to heat, *(c)* no purulent discharge, *(d)* no abnormality of the reproductive tract detectable by palpation per rectum, *(e)* not more than ten years old, and *(f)* Bang's-negative. One cow was obtained from each of 56 herds, two cows from each of four herds, and five cows from one herd.

The 69 experimental repeat-breeders included Holstein, Guernsey, Jersey, and Brown Swiss dairy cattle. Fifty-five had calved at least once and 14 were nonparous heifers. Each had been bred from four to 21 times before the experimental service; the mean was 7.2 services. All except a very few of the services on the farms were by artificial insemination, with semen diluted with milk or egg yolk–citrate and containing antibacterial agents.

The cows were trucked to a University barn near Madison as they were selected. Heat-checks were made twice daily, a cow being considered in heat when it stood for mounting by other cows. The animals were artificially inseminated approximately 12 hr. after estrus was detected, with 1 ml. of day-of-collection semen diluted with milk or egg yolk–citrate and containing sulfanilamide, penicillin, and streptomycin. The semen was obtained from unselected stud bulls used regularly in artificial breeding, and was deposited in the body of the uterus. Two heifers failed to show signs of estrus in connection with ovulation, soon after being brought to the experimental barn. They were slaughtered

15 days after the time of ovulation, estimated from the routine sequence of palpation of the ovaries per rectum.

The reproductive organs were palpated at least once between eight and 15 days postbreeding, to determine the presence of a corpus luteum and the tone of the uterus. The majority of animals were slaughtered at the end of 16 elapsed days from the beginning of heat; three were slaughtered at 17 days, and 19 animals that were first noticed in heat in the evening were slaughtered 15⅔ elapsed days after the first signs of estrus.

The animals were slaughtered at the University meats laboratory. After being stunned, each was bled while lying on its side rather than while hanging from a hoist. This procedure was followed to reduce the possibility of bacterially contaminated vaginal mucus draining through the cervix into the uterus. Immediately after the animal was dead, it was rolled on its back and the abdomen opened. The uterine horn on the side opposite the ovary which contained the corpus luteum was ligated near the tubo-uterine junction, to prevent possible drainage through the oviduct of peritoneal fluid which might have been contaminated when the peritoneal cavity was opened. The ovaries and reproductive tract anterior to the vagina were then removed from the animal. Vaginal mucus samples were obtained from the last 35 animals on the project; the ventral pelvic bone was cut in these cattle, to facilitate removal of a greater length of the vagina. A ligature was placed around the vagina 4 to 6 in. posterior to the cervix before removal of the reproductive tract, to prevent the escape of vaginal and cervical mucus. The organs were handled throughout in such a manner as to minimize the chances of drainage from the cervix and vagina into the uterus.

The genital organs were examined carefully for any anatomical abnormalities which might have interfered with fertilization. The two uterine horns were then separated by dissecting the intercornual ligament between the external and internal bifurcations; both uterine horns were ligated at the internal bifurcation and excised from the remainder of the tract. The time interval from the stunning of the animal to this point was approximately 15 min.

The uterine horn on the side opposite to that of ovulation was used for the bacteriological study; the other uterine horn was flushed to determine the presence of an embryo. It was assumed that any microorganisms present would likely be in both uterine horns, rather than restricted only to the horn on the side of ovulation. The uterine horn which was to be used for the bacteriological study was placed in a sterile container and taken immediately to the Bacteriology Building, and the work there carried on in a sterile room.

LABORATORY WORK

A small area on the uterine serosa was sterilized by searing with a hot spatula, and 15 ml. of 0.1% tryptone broth was injected into the uterine lumen by puncture of the uterine wall in the seared area with a 19-gauge hypodermic needle. The needle hole was closed by searing with a hot spatula, and the uterine horn

manipulated vigorously, to suspend in the solution any microorganisms which might be in the lumen. The fluid was then forced into the tubal end of the horn, an area seared, and the fluid withdrawn by syringe and hypodermic needle for bacteriological study.

Bacteria could possibly live and multiply within the tissues of the endometrium and cause damage leading to embryonic death, without actually coming in contact with the embryo. Such bacteria might be overlooked by culturing only washings from the uterine lumen. In this work, therefore, bacteriological studies were made of triturated endometrial tissue of the last 35 animals. After the tryptone broth was removed, the uterine horn was placed in a sterile box and an area approximately 2 in. long and 1 in. wide at the level of the external bifurcation was seared with a large spatula. The endometrium was exposed by carefully removing the serosa and myometrium in the seared area. All of the endometrial tissue was excised for a length of approximately 2 in. and placed in a Petri dish, where the most vascular caruncular areas and excess connective tissue underlying the endometrium were removed. The endometrial tissue was cut into small pieces with scissors and triturated aseptically in a TenBroeck grinder in 3 ml. of 0.1% tryptone broth. After the tissue was thoroughly ground, the fluid was decanted and used for bacteriological culture.

Various culture media were used to provide a wide range of growth conditions. The media and the reasons for the use of each were:

(a) Blood agar—Difco blood agar base plus 5% citrated bovine blood was used. Onto the surface of the medium was placed 0.1 ml. of the fluid to be cultured and this was spread with a sterile glass rod. This medium was used as one likely to grow such organisms as staphylococci, streptococci, diphtheroids, brucellae, or *V. fetus.*

(b) EMB agar—Difco EMB agar was used and cultures were prepared by the surface-spread technique. This medium was used to detect the presence of coliforms and related pathogens.

(c) Albimi brucella broth—To this medium was added 0.1% agar to make a semisolid nutrient solution that is known to support the growth of the brucellae and *V. fetus.* This was inoculated with 0.1 ml. of fluid (obtained as just described).

(d) Thiol medium—This Difco medium was supplemented with 0.4% agar and 0.05 g. per liter of glutathione (reduced), according to the method of Hughes and Gilman *(7)*. This medium was inoculated by stabbing it with a loopful of fluid, and was used as one likely to obtain growth of *V. fetus* and anaerobes.

(e) PPLO medium—Difco PPLO agar supplemented with 1% Bacto-PPLO serum fraction was used to prepare plates for the possible detection of pleuropneumonia-like organisms. This medium was inoculated by spreading 0.1 ml. of the fluid over the surface.

TABLE 1
Media and culture environments used

Medium	Number of cultures per sample in each environment		
	Air	Increased CO_2 Decreased O_2	Anaerobic
Blood agar	3 Fluid[a] 3 Controls	3 Tissue[b] 3 Fluid 3 Controls	3 Fluid 3 Controls
EMB	3 Fluid 3 Controls	3 Fluid 3 Controls	3 Fluid 3 Controls
Albimi brucella broth	3 Fluid 3 Controls	3 Fluid 3 Controls	3 Fluid 3 Controls
PPLO	4 Fluid 4 Controls	.....	.....
Thiol	.....	6 Fluid 5 Tissue	.....

[a] Fluid—those with fluid from uterus.
[b] Tissue—from triturated uterine tissue.

A large number of plates and tubes of each medium was made from the fluids collected from each uterus. The plates and tubes were incubated at 37° C. under three different environmental conditions; i.e., in air, in 15% CO_2 and reduced oxygen, and in an anaerobic jar. In all cases, a large number of uninoculated control plates and tubes were incubated, to insure that any growth obtained from the fluids did not arise as contamination, during preparation of the cultures. From each animal there were 25 plates for cultures plus 22 control plates, and 20 tubes for culture plus 9 control tubes. This distribution is shown (Table 1). In addition to the cultural tests just described, the fluids recovered from the uteri were examined under the microscope by the use of stained smears of the material. Vaginal mucus from the last six cows in the trial was inoculated into a special selective medium, Kuzdas and Morse *(8)*, for *V. fetus*.

A blood sample was taken from each animal immediately prior to slaughter. The blood was allowed to clot, the serum removed and frozen for subsequent agglutinin-lysis test for leptospirosis.

The *V. fetus* tube-agglutination test was performed on two vaginal mucus samples from each of the last 35 animals used in the study. The samples were taken at from 11 to 13 days postbreeding and shortly after slaughter. Each sample was tested with two antigens. The method used in obtaining the vaginal mucus and in performing the tests was essentially that described by Hughes *(6)*. In addition, a sample of the uterine washings of each of the 35 animals was tested in the same manner.

Soon after the animal was slaughtered, the uterine horn on the side of ovulation was flushed with about 15 ml. of physiological saline solution. Each embryo that was recovered was measured, then examined under a low-power microscope for normality and measurement of the embryonic disc.

RESULTS

Organisms were cultured from the uterine washings of eight (11.6%) of the 69 repeat-breeder cattle included in the study. A yeast, *Candida krusei*, was recovered from four of the infected uteri, a paracolon (family *Enterobacteriaceae*) from one, an *Escherichia* species from one, a *Pseudomonas* species from one, and *Brucella abortus* from another, the latter animal being Bang's-negative at the time of purchase.

The experimental animals were classified according to the presence or absence of: (1) mechanical obstructions of the reproductive tract, (2) pus in the uterus at the time of slaughter, (3) an embryo, and (4) isolation of microorganisms from the uterus (Table 2).

Five of the 69 animals had mechanical obstructions or abnormalities of the reproductive organs which might have interfered with fertilization. One animal had a bilateral hydrosalpinx, one had adhesions of the bursa to both ovaries, two had adhesions of the infundibulum to the mesosalpinx, and one had a completely double cervix. None of these five animals had an embryo in the uterus and no microorganisms were cultured from the uterine washings.

TABLE 2

Classification of experimental animals based on the presence[a] or absence of (1) mechanical obstruction of the reproductive organs, (2) pus in the uterus, (3) an embryo, and (4) microorganisms isolated

Mechanical obstruction	Accumulated pus	Embryo	Microorganism isolated
5+	0+	.....	.....
	5—	5—	5—
64—	7+	1+	1—
		6—	4+[b]
			2—
	57—	30+	3+[c]
			27—
		25— (plus two not bred)	1+[d]
			26—

[a] The presence or absence of each factor is indicated in the table by a + or — sign. The numeral preceding each sign is the number of animals in the classification.

[b] One isolation each of *Candida krusei*, *Pseudomonas*, *Escherichia*, and a paracolon.

[c] Two isolations of *C. krusei*, one of *Brucella abortus*.

[d] *C. krusei*.

The other 64 animals had reproductive tracts which were normal anatomically. Gross inspection of the uterus and observation of the uterine washings

revealed that seven of the 64 had an accumulation of pus in the uterine lumen. The quantity of the pus was in the order of 1 ml. or less in each uterine horn of six of these cows; the uterus of one cow contained approximately 10 ml. of thick, gummy pus in each horn. Neither the quantity of pus in the uterine lumen nor the tone of the uterus was such that the condition could be detected by rectal palpation of the uterus prior to slaughter. An embryo was recovered from one of these seven uteri; the washings from this uterus and from two others which contained small amounts of pus were sterile when cultured. Four of the seven uteri containing pus were infected; *C. krusei* was isolated from one, an *Escherichia* species from one, a paracolon from one, and a *Pseudomonas* species from the uterus containing the large quantity of pus.

Fifty-seven of the anatomically normal uteri showed no macroscopic accumulation of pus. Embryos were recovered from 30 of these uteri, 27 of which were sterile bacteriologically. *C. krusei* were isolated from two of the uteri which contained embryos, and *B. abortus* from one. Embryos were thus recovered from three of the eight uteri from which organisms were isolated. Phagocytized gram-positive cocci were seen in the washings of one uterus which contained an embryo, but the fluid was sterile when cultured. Embryos were not recovered from 27 of the anatomically normal uteri which contained no pus. Two of these animals had not been bred experimentally, and no organisms were cultured from their uterine washings when they were killed approximately 16 days post-ovulation. Of the other 25 uteri from which embryos were not recovered, 24 were sterile bacteriologically, and *C. krusei* was recovered from one. One of the cows whose uterus was sterile bacteriologically gave a positive vaginal-mucus tube-agglutination test for *V. fetus* on both vaginal-mucus samples. Of the 35 animals tested in this manner, this was the only one which gave a positive test. This particular cow was obtained from a herd in which vibriosis has never been recognized as a problem.

In the case of the four uteri from which *C. krusei* was isolated, the organism was seen in large numbers on microscopic examination of the uterine washings of three of the animals.

An agglutination-lysis test for leptospirosis was performed on blood serum from each of the animals. All of these tests were negative. These tests were performed along with tests on serum samples from a Leptospirosis project from which positive samples were obtained. Thus, we were confident that the agglutination-lysis test was working properly.

Fourteen of the repeat-breeders were nonparous heifers. Three of these heifers had mechanical obstructions which might have interfered with fertilization; one had adhesions of the infundibulum to the mesosalpinx, one had ovarian adhesions, and one had a double cervix. These heifers had no embryos. Two of the heifers were those animals which were not bred. Three of the nine other heifers had an embryo in the uterus and six did not. Microorganisms were not recovered from the uterus of any heifer.

The results of this study fail to indicate that infectious agents in the uterus

cause an appreciable amount of the embryonic death which occurs shortly after 16 days postbreeding in the clinically normal repeat-breeder cow. Again, it must be emphasized that this is a selected kind of cow, and that the observations in this study have little bearing on the broad role of microorganisms in lowered fertility in cattle. Most of the studies previously reported either have ignored the clinical variations in the cows under consideration or have intentionally limited the study to clinically abnormal animals. The main reason for the present results differing from others probably rests on this difference in animals.

The possibility also should not be completely ignored that organisms actually were present which were of a kind that produced little or no inflammation [uterine condition described by Simon and McNutt *(12)*] and which could not be detected by our methods. Then, too, the possibility of there being undetermined carry-over effects from earlier infections, which interfered with the physiological ability of the uterus to support a pregnancy, probably deserves some inquiry. Logically, these two possibilities must be kept in mind, in dealing with the general problem. For the present, however, there seems to be little evidence that infectious agents play a major role in the lowered fertility of cows such as studied here.

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EFFECT OF PREVIOUS RATION ON LACTATION RESPONSE TO SUBSTITUTING CORN FOR ALFALFA ACCORDING TO NET ENERGY AND TOTAL DIGESTIBLE NUTRIENT SYSTEMS OF ESTIMATION[1]

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SUMMARY

The lactation response to the substitution of 10 lb. alfalfa with 7.0 or 5.7 lb. corn based, respectively, on the TDN or NE system was studied, in a double-reversal trial, with 24 cows previously fed equivalent therms NE per unit of live weight from either of two basal rations: 100% alfalfa or 80% alfalfa plus 20% corn. Basing the substitution on the TDN system resulted in greater energy intake than the NE system, 0.99 therm and 0.94 lb. TDN. Across both basal rations, the TDN basis of substitution resulted in significant increases in average daily milk production, 0.89 lb. milk, 0.025 lb. fat, and 0.73 lb. FCM, and a 0.10 decrease in the fat percentage, over the NE basis. Assessing the advantage of the TDN basis of substitution for each basal ration fed, the cows previously fed the 80% alfalfa plus 20% corn basal ration failed to respond; whereas, those previously fed the 100% alfalfa basal showed the following significant advantages: milk 1.56 lb., fat 0.051 lb., and FCM 1.39 lb. When 10 lb. alfalfa were replaced with equivalent NE from corn, milk production increased 1.55 lb. and FCM 0.94 lb. for the 100% alfalfa basal. No significant changes for the 80% alfalfa plus 20% corn basal were observed and across both basal rations, a slight increase in pounds of milk.

The adequacy or inadequacy of an all-alfalfa hay ration for lactation in dairy cattle is controversial. Huffman and Duncan *(14)*, after feeding an all-alfalfa ration, which was considered a "depletion" ration, until milk production and live weight changes became minimal, replaced a portion of the alfalfa with equivalent amounts of total digestible nutrients from corn. Milk production increased and this was attributed to unidentified lactation factor(s) contained in the corn. These same workers, as well as others *(9, 27)*, have obtained similar lactation responses with other concentrates, immature hays, and silages. In contrast, others *(15, 24)*, under somewhat similar experimental conditions, have noted increases

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in milk production, but accounted for these increases on the basis of greater net energy intake. When net energy was held constant in substituting corn into an all-alfalfa ration, no increase in milk production was observed. Loosli *et al. (19)* essentially have confirmed these observations.

The present study was undertaken to determine the lactation response to the substitution of corn for alfalfa, on an estimated net energy basis and on an estimated total digestible nutrient basis, as affected by previous freeding of equivalent net energy per unit of live weight from either a 100% alfalfa basal ration or an 80% alfalfa plus 20% corn basal ration.

EXPERIMENTAL PROCEDURE

Animals and rations. Twenty-four cows—12 Guernseys, 4 Holsteins and 8 Jerseys—were placed on experiment during the period of January to October, 1953, on either the 14th or 35th day postpartum. Within a breed, groups of four cows were selected for similarity of age and previous calvings (Table 1). Within

TABLE 1

Average age, previous lactations, days in milk, production of 4% FCM, live weight and days in gestation of the experimental groupings with their standard errors

	Basal ration			
	100% Alfalfa		80% Alfalfa + 20% corn	
	Sequence of substitution			
	NE-TDN-NE	TDN-NE-TDN	NE-TDN-NE	TDN-NE-TDN
Years of age at end of depletion period	3.7 ± 0.3	4.0 ± 0.4	3.5 ± 0.5	4.8 ± 0.9
Previous lactations	0.7 ± 0.3	1.5 ± 0.4	0.7 ± 0.3	1.8 ± 0.8
Days in milk at end of depletion period	95 ± 4	94 ± 6	94 ± 6	95 ± 5
Production of 4% FCM at end of depletion period	24.4 ± 3.5	25.0 ± 2.4	25.1 ± 2.2	30.3 ± 2.9
Live weight at end of depletion period	850 ± 93	928 ± 108	870 ± 68	965 ± 88
Days in gestation at end of experimental period	64 ± 18	74 ± 17	27 ± 24	54 ± 23

each set of four, individual cows were assigned according to a previously randomized allotment to one of two basal rations, 100% alfalfa, or 80% alfalfa plus 20% corn; and to one of two sequences of methods of substitution of corn for alfalfa, namely, estimated net energy (NE) and estimated total digestible nutrients (TDN), in the following sequences: NE-TDN-NE or TDN-NE-TDN, designated as Sequence A and Sequence B, respectively.

Cows on the 100% alfalfa ration were fed 3.0 lb. of dry matter of alfalfa per 100 lb. of live weight per day. Cows on the 80% alfalfa plus 20% corn ration were fed at such a rate that the ration equaled the 100% alfalfa ration in NE content. NE was calculated by the formula of Moore *et al. (22)*, derived

from Forbes' data, based on TDN values calculated from proximate analysis according to Schneider *et al. (26)*. During this feeding period, the depletion period, which continued through the 70th day, the ration allowance was adjusted for live weight on the 15th and 57th days.

On the 71st, 99th, and 127th days, the ration allowance of the cows on the 100% alfalfa basal was reduced to 95%, 90%, and 85%, respectively, of that fed during the 57th to 70th days. Cows on the 80% alfalfa plus 20% corn basal were fed accordingly equivalent amounts of NE. On the 85th day, 10 lb. of alfalfa was replaced with corn on either the NE or TDN basis, according to the assigned sequence. This was repeated on the 113th and 141st days. This feeding period followed a double change-over design *(6)* consisting of three 28-day periods. Each of these was made up of a 14-day standardizing period to establish lactation level, and a 14-day comparison period to test for differences in response to the two systems of substitution.

During the entire experiment, all cows received 0.3 lb. steamed bone meal daily, Guernseys and Jerseys 0.1 lb. iodized salt daily, and Holsteins 0.2 lb.

The alfalfa hay which was second-cutting, field-cured, and New York State grown, was chopped at a setting of ¾-inch. Samples of the hay, taken at 4-wk. intervals, graded on the average U.S. No. 2.2 alfalfa. They were composed of 5.1% foreign matter and the remainder of 93.0% alfalfa and 7.0% grasses and other legumes, and had 68.2% color and 28.5% leafiness. The corn, U.S. No. 1 dent, was fed coarsely ground. Average chemical composition of these feeds, as well as calculated NE and TDN content, are contained in Table 2.

Observation and analyses. All feeds, fed and refused, and milk yield were weighed to the nearest 0.1 lb. twice daily. Live weights were recorded to the nearest pound on the first, sixth and seventh day of each seven-day period.

Milk samples of the A.M. and P.M. milkings were taken every seven days during the depletion period, and daily during the successive standardizing and comparison periods, for fat determination by the Babcock method *(1)*. Four per cent fat corrected milk (FCM) was determined by the formula of Gaines *(12)*. Samples of feeds, fed and refused, were taken at each feeding and composited each seven-day period for proximate analysis *(1)*. TDN content of the weighbacks was computed from proximate analysis by the method of Schneider *et al. (25)* and NE as previously described.

The data were analyzed as outlined in the Statistical Appendix. In the analysis, the effect of advancing lactation upon the treatment responses was eliminated, based on each cow's linear decline. Only data for the last seven days of each standardizing and comparison period were used in the analysis. In one cow in the first replicate, missing values for the second standardizing and comparison period were derived, owing to a miscalculation of its feed allowance.

RESULTS

Average daily observed values of the feed intakes, the milk production criteria, and live weight changes for each standardizing and comparison period by

TABLE 2

Average proximate analysis, estimated total digestible nutrients, and net energy of feeds with their standard errors

Feed	Composition									
	Moisture	Crude protein	Crude fiber	Nitrogen-free extract	Ether extract	Ash	Ca [c]	P [c]	TDN	Therms NE
	(%)									
Alfalfa [a]	9.1 ±0.2	16.2 ±0.1	26.0 ±0.2	39.7 ±0.2	1.9 ±0.0	7.1 ±0.1	1.36	0.23	56.4 ±0.2	46.7 ±0.3
Corn [b]	11.6 ±0.2	8.6 ±0.0	1.7 ±0.0	72.8 ±0.4	4.0 ±0.0	1.3 ±0.0	0.02	0.28	79.2 ±0.2	80.9 ±0.2
Steamed bone meal [c]	2.65	.....	.....	.....	.....	.....	29.30	14.02	.....	.....

[a] Average of 94 samples.
[b] Average of 81 samples.
[c] Average of two samples.

experimental subgroupings are shown (Table 3). Responses to the substitution of corn for 10 lb. alfalfa, using either the TDN or NE system as affected by the previously fed basal ration, are given (Table 4). The values for average daily intake of therms of NE and pounds of TDN were based on each cow's actual feed intake and feed refusal and the proximate analyses for the particular week.

Feed intake. Basing the substitution of 10 lb. of alfalfa on the TDN system of evaluation resulted in the feeding of 7.0 lb. of corn; whereas, 5.7 lb. of corn were fed when the substitution was based on the NE system (Table 3). This resulted in an average daily advantage of 0.99 therm and 0.94 lb. TDN for the TDN substitution, when averaged over both basal rations (Table 4) and similar values for each basal ration.

It was anticipated that the NE and TDN intakes would remain constant when the substitution was based on the corresponding system. However, small differences were observed (Table 4) which most likely were due in part to variation in the weekly proximate analysis and in part to small feed refusals.

In the case of the 80% alfalfa plus 20% corn basal, substituting corn for alfalfa, especially in the last period, resulted in relatively low intakes of hay. However, no abnormally low butterfat tests were observed, even with intakes of 1 to 3 lb. per cow daily.

Milk production. When averaged over both basal rations, the advantage of basing the substitution of corn for alfalfa on the TDN system was a significant increase in production of milk, FCM, and fat, and conversely in significant decreases in the fat percentage (last row, Table 4). Such a response might have been anticipated from the previously indicated increases in energy intake. Examination of the lactation responses as affected by the basal ration fed yielded the following results: Substituting corn for alfalfa in the 100% alfalfa basal showed a very significant advantage for the TDN basis of substituting over the NE basis; whereas, no advantage was noted for the TDN basis when the substitution was made with the 80% alfalfa plus 20% corn basal. Even though the energy intake showed essentially the same advantage for both basals, 1.01 and 0.97 therms and 0.96 and 0.92 lb. TDN, respectively, for the TDN basis of substitution, the animals previously fed 100% alfalfa ration increased their production by 1.39 lb. FCM, while the animals fed the 80% alfalfa + 20% corn ration failed to respond. Of further interest was the observation that animals previously fed the 100% alfalfa ration responded to the substitution of corn on the NE basis, in spite of an intake of therms NE which remained essentially the same, by producing 0.94 lb. FCM more. In general, pounds of milk and fat showed the same trends as discussed for FCM, and the response for the fat percentage followed an opposite trend.

Live weight changes. The live weight data were analyzed in terms of average daily change in live weight during the last seven days of each standardizing and comparison period, rather than in terms of actual live weight. The use of actual live weights in change-over trials may possibly mask treatment differences, because of changes in digestive fill from period to period. In general, responses

TABLE 3

Average daily observed feed intakes, milk production, and live weight changes for each period

Criteria	Experimental groupings: Basal ration	Sequence of substitution[a]	Experimental period: I Std.	I Comp.	II Std.	II Comp.	III Std.	III Comp.
Alfalfa fed *(lb.)*	100% Alfalfa	A	26.9	16.9	25.4	15.4	24.1	14.1
		B	29.6	19.6	28.0	18.0	26.4	16.4
	80% Alfalfa + 20% corn	A	18.8	8.8	17.8	7.8	16.7	6.7
		B	21.0	11.0	19.5	9.5	18.8	8.8
Corn fed *(lb.)*	100% Alfalfa	A	0	5.7	0	7.0	0	5.7
		B	0	7.0	0	5.7	0	7.0
	80% Alfalfa +20% corn	A	4.7	10.4	4.4	11.4	4.2	9.9
		B	5.2	12.2	4.8	10.5	4.7	11.7
Total NE intake *(therms)*	100% Alfalfa	A	12.6	12.5	11.7	12.6	11.3	11.2
		B	13.5	14.5	12.8	12.6	12.6	13.3
	80% Alfalfa + 20% corn	A	12.6	12.5	12.0	12.8	11.1	11.3
		B	13.8	15.0	12.9	12.8	12.4	13.4
Total TDN intake *(lb.)*	100% Alfalfa	A	15.2	14.1	14.2	14.0	13.6	12.5
		B	16.4	16.3	15.6	14.3	15.0	14.8
	80% Alfalfa + 20% corn	A	14.2	13.2	13.7	13.4	12.7	11.8
		B	15.8	15.9	14.8	13.6	14.2	14.0
Milk *(lb.)*	100% Alfalfa	A	23.3	24.1	20.8	22.8	18.0	19.2
		B	23.8	25.2	21.0	20.2	18.1	19.2
	80% Alfalfa + 20% corn	A	23.2	22.4	21.0	20.0	18.1	17.9
		B	27.5	26.3	24.6	23.3	22.1	21.5
Fat *(%)*	100% Alfalfa	A	4.5	4.3	4.5	4.3	4.6	4.5
		B	4.5	4.2	4.4	4.4	4.5	4.4
	80% Alfalfa + 20% corn	A	4.7	4.8	4.9	4.9	5.2	5.2
		B	4.8	4.7	4.7	4.8	4.9	5.0
Fat *(lb.)*	100% Alfalfa	A	1.0	1.0	0.9	0.9	0.8	0.8
		B	1.0	1.0	0.9	0.9	0.8	0.8
	80% Alfalfa + 20% corn	A	1.0	1.0	1.0	0.9	0.9	0.9
		B	1.3	1.2	1.1	1.1	1.0	1.0
4% FCM *(lb.)*	100% Alfalfa	A	24.4	24.4	21.6	23.2	20.0	20.1
		B	25.0	25.6	22.0	21.0	19.1	20.1
	80% Alfalfa + 20% corn	A	25.1	24.4	23.0	22.1	20.7	20.4
		B	30.3	28.6	26.6	25.3	24.7	24.1
Change in live weight *(lb.)*	100% Alfalfa	A	+0.1	+0.7	+1.0	0.0	+0.2	+0.2
		B	+0.2	+0.5	+1.2	+0.2	−1.3	+0.7
	80% Alfalfa + 20% corn	A	0.0	−1.8	+0.7	−1.8	+0.4	+1.1
		B	−0.7	−1.2	−0.5	+0.4	+1.5	−0.1

[a] Sequence A—NE-TDN-NE; Sequence B—TDN-NE-TDN.

in changes of live weight to the substitution of corn for alfalfa were not significant. However, of interest is the observation that there was no advantage for substituting on the basis of TDN in the case of the 100% alfalfa basal, 0.01 lb., while there was a pronounced loss of live weight for the 80% alfalfa plus 20% corn basal, −1.40 lb., which is the same trend as observed for the response of FCM.

Two factors which may have influenced the results of this study are that protein intake, either as per cent of ration or total daily intake, was not equalized between basal rations or methods of substitutions, and that the proportion of roughage to concentrate was not held constant within basals upon substitution. Unfortunately, these effects, if real, were not measurable in this study.

Calculated NE values of alfalfa. To explore whether a difference existed in the utilization of the alfalfa fed in this study, between the 100% alfalfa basal group and 80% alfalfa plus 20% corn basal, NE values were computed for the alfalfa for each basal. Only data from the three standardizing periods, when the basals were fed on an equivalent NE intake per unit of live weight, were utilized. In these calculations, 6.0 therms per 1,000 lb. live weight to the 0.7 power were allowed for maintenance and 4% FCM at 0.30 therm per pound. The NE value of corn was based on the particular week's proximate analysis and derived as previously described. Based on the individual cow's requirement for maintenance and production, the net energy value of the alfalfa was found to be 45.53 therms per 100 lb. when fed alone, as in the 100% alfalfa basal, and 49.92 therms per 100 lb. when fed in conjunction with corn, as in the 80% alfalfa plus 20% corn basal. Although this is not very conclusive evidence, it indicated that the nutritive value of the alfalfa may have been increased by the addition of limited amounts of corn.

DISCUSSION

This study substantiated essentially the results of Huffman *et al. (14)* and other workers *(9, 27)* that, when part of an all-hay ration is substituted with a concentrate on an equivalent TDN basis, milk production will increase. That such an increase appeared owing to increased intake of NE, as pointed out by Saarinen *(24)* and Irvin *(15)*, was also substantiated. However, in this study not all the increase appeared to be due to the increased NE intake, inasmuch as the cows fed 100% alfalfa previously showed increased milk production when 10 lb. of alfalfa was replaced with equivalent amounts of NE from corn, which is in agreement with the study of Loosli *et al. (19)*. The above observation, and the fact that cows fed the 80% alfalfa plus 20% corn ration failed to respond to the increased energy, when corn was substituted on the TDN rather than the NE basis, suggested the possibility that the effective energy content of a ration may to some degree be affected by the proportion of roughage to concentrate it contained, or by other factors, such as protein intake and so on.

The TDN basis of estimation of the nutritive value of feedstuffs has long been suspected to change with the proportion of roughage to concentrates; Beach *(3)* in 1906 showed that 4.5 lb. of TDN from corn was equivalent to 7.1 lb. of TDN from hay for the maintenance of steers, when both feedstuffs were fed by

TABLE 4

Responses to the substitution of corn for alfalfa of feed intakes, milk production, and live weight changes

Response to substituting corn for alfalfa	NE intake	TDN intake	Milk	Fat	Fat	FCM	Live weight
	(therms/day)	*(lb/day)*	*(lb/day)*	*(%)*	*(lb/day)*	*(lb/day)*	*(lb/day)*
100% alfalfa basal							
TDN basis	1.16	0.19	3.11	−0.24	0.083	2.33	0.28
	(0.27)[a]	(0.24)	(0.81)	(−0.12)	(0.034)	(0.74)	(1.30)
NE basis	0.15	−0.77	1.55	−0.13	0.032	0.94	0.27
	(0.27)	(−0.24)	(0.81)	(−0.12)	(0.034)	(0.74)	(1.30)
Advantage for TDN over NE basis	1.01	0.96	1.56	−0.11	0.051	1.39	0.01
	(0.30)	(0.29)	(0.71)	(−0.14)	(0.034)	(0.77)	(1.56)
80% alfalfa basal							
TDN basis	1.31	0.25	0.42	−0.12	−0.003	0.10	−1.98
	(0.27)	(0.24)	(0.81)	(−0.12)	(−0.034)	(0.74)	(−1.30)
NE basis	0.34	−0.67	0.22	−0.04	−0.002	0.03	−0.58
	(0.27)	(−0.24)	(0.81)	(−0.12)	(−0.034)	(0.74)	(−1.30)
Advantage for TDN over NE basis	0.97	0.92	0.20	−0.08	−0.001	0.07	−1.40
	(0.30)	(0.29)	(0.71)	(−0.14)	(−0.034)	(0.77)	(−1.56)
Average of both basals							
TDN basis	1.23	0.22	1.77	−0.18	0.040	1.22	−0.85
	(0.19)	(0.17)	(0.58)	(−0.08)	(0.024)	(0.52)	(−0.92)
NE basis	0.24	−0.72	0.88	−0.08	0.015	0.49	−0.16
	(0.19)	(−0.17)	(0.58)	(−0.08)	(0.024)	(0.52)	(−0.92)
Advantage for TDN over NE basis	0.99	0.94	0.89	−0.10	0.025	0.73	−0.69
	(0.21)	(0.20)	(0.50)	(−0.10)	(0.024)	(0.54)	(−1.10)

[a] Response needed for significance at $P = 0.05$.

themselves. Also, the NE basis of estimation may not be additive as the proportion of hay to corn is changed. Evidence for this is provided by Forbes *(11)*, who found that the NE value of corn when fed alone was less than when corn and alfalfa hay were fed together. Indirect evidence in feeding experiments with lambs, calves, and steers indicates there is possibly an optimum proportion of roughage to concentrates for maximum growth and gross feed efficiency *(8, 10, 16)*. It would, therefore, appear that such might be the case for lactation response in dairy cows, as is suggested by the data of Martin *et al. (21)*, even though the curvilinearity in their data was not statistically significant.

Considerable controversy currently exists as to the most applicable predictor of the nutritive value of various feedstuffs for dairy cattle *(4, 7, 17, 22, 23, 28)*. These studies and reviews have involved not only the use of TDN and NE but also other systems such as starch equivalent, productive energy, metabolizable energy, and digestible energy. Most of these have been concerned with comparisons among systems or establishment of nutritive values over very limited ranges of combinations of roughage and concentrates. The aforementioned studies (as well as the one reported herein on the substitution of corn for alfalfa) cover only a limited range of possible combinations of roughages and concentrates, and at only a very narrow range of nutritive intakes. Therefore, it would appear that additional data need to be collected, with lactating dairy cows, at several fixed planes of nutrition, and at each plane of nutrition different combinations of hay to grain covering the possible range from all roughage to all grain. A recent study by Bloom *et al. (5)* has provided some preliminary information of this type. Such data possibly would allow present systems of feed evaluation to be used, but modified with a factor(s) descriptive of the type of ration fed and the plane of nutrition. The establishment of a good possible functional relationship of milk production to type of ration and plane of nutrition awaits collection of appropriate data *(2, 13)*. Such data would also lend themselves to economic interpretation of optimum input-output price ratios and rates of substitution of feedstuffs.

Accurately to evaluate feed utilization by the lactating dairy cow, both milk production and body composition changes need to be evaluated simultaneously *(18)*. Because methods to estimate changes in body composition and the contribution of rumen fill to live weight are now in the developmental stage, most studies including the one reported here are as yet unsuitable to evaluate successfully and simultaneously the two measures of energy utilization, milk production and body composition.

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STATISTICAL APPENDIX

The Analysis Problem

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The experimental design of the study reported here may be represented as follows:

Period	Treatment sequence			
	1	2	3	4
1	a	a	a'	a'
2	b	c	b'	c'
3	a	a	a'	a'
4	c	b	c'	b'
5	a	a	a'	a'
6	b	c	b'	c'

In the table:

a, a' represent the two basal rations used, i.e., 100% alfalfa, and 80% alfalfa + 20% corn, respectively.

b, b' represent the substitution of corn for alfalfa in the basal rations on the NE basis.

c, c' represent the substitution of corn for alfalfa in the basal rations on the TDN basis.

The basic design was replicated six times.

The comparisons b *vs.* c and b' *vs.* c' presented no problem in analysis, because the observations on a and a' could be neglected and the data analyzed, as for switch-back trials by the method of Brandt *(6)*. For the comparisons of a with b and c, and of a' with b' and c', however, a method of analysis had to be formulated. The principal problem was to make the latter comparisons fair.

A straight-out comparison of corn and alfalfa by the use of simple averages could be biased, because a and a' did not appear in the same experimental periods as did b, b', c, or c'. One factor which, under the treatment arrangement used, could favor a and a' is the decline in production as lactation advances. Correction for this was made in the analysis by assuming a linear decline different for each cow. Another factor which could have caused bias, if all replications of the basic design had been started at the same time, is the shift in general environment from one period to another. Because the several replications and usually the several cows in a replication were started at different times, however, it was assumed that the treatments favored changed from cow to cow in an essentially random fashion and that no bias was introduced. Examination of the data indicated that this assumption was fairly safe to make in the present experiment. In general, however, the assumption of no period effects is not a safe one, and a design not requiring such an assumption is preferable *(20)*.

Comparison of alfalfa with corn.

The first step was to compute the quantity

$$P = 5y_1 + 11y_2 - 8y_3 + 8y_4 - 11y_5 + 5y_6$$

for each cow, where $y_1, y_2, \ldots, y_6$ are the performances during periods 1,2, . . . , 6, respectively. For the cow with the missing data, P was taken as the average of the P's for the other cows on the same sequence. It is seen that P is clear of the linear time trend, because

$$-5(1) + 11(2) - 8(3) + 8(4) - 11(5) + 5(6) = 0.$$

In order to outline the analysis, let

$P_{ij} = P$ value for the jth cow on the ith sequence ($i = 1,2, \ldots, 4$; $j = 1,2, \ldots, n$; here $n = 6$)

$G_i =$ sum of the P values on the ith sequence.

The error mean square on the per-observation basis was computed as

$$s_P^2 = \frac{1}{420(4)(n-1)} \left[\sum_i \sum_j P_{ij}^2 - \sum_i \frac{G_i^2}{n} \right]$$

in which $4(n-1) =$ degrees of freedom for error. Here d.f. $= 4(6\text{-}1) = 20$, basically, but because of the missing value for one cow, d.f. $= 19$, actually.

The advantage for corn substitution averaged over both basal rations *(D)* and its standard error *(s_D)* are given by

$$\frac{G_1 + G_2 + G_3 + G_4}{96n} \text{ and } \sqrt{\frac{35s_P^2}{192n}}$$

respectively. The advantages for corn in the 100% alfalfa ration and in the 80% alfalfa ration are given by

$$d(100) = \frac{G_1 + G_2}{48n} \text{ and } d(80) = \frac{G_3 + G_4}{48n}$$

both with standard error, $s_D\sqrt{2}$. The additional advantage that corn substitution in the 100% alfalfa ration has over corn substitution in the 80% alfalfa ration is

$$d(100\text{-}80) = \frac{G_1 + G_2 - G_3 - G_4}{48n}$$

with standard error $2s_D$.

Comparison of TDN substitution with NE substitution:

Following Brandt (1938), the quantity

$$Q = y_2 - 2y_4 + y_6$$

was computed for each cow. For the cow with missing data, Q was taken as the average of the Q's for the other cows on the same sequence. Q is orthogonal to the P previously computed. It is also clear of linear time trends, since

$$1(2) - 2(4) + 1(6) = 0.$$

To describe the computations, let

$Q_{ij} = Q$ value for the jth cow on the ith sequence

$H_i =$ sum of the Q's on the ith sequence

The error mean square is given by

$$s_Q^2 = \frac{1}{6(4)(n-1)}\left[\sum_i \sum_j Q_{ij}^2 - \sum_i \frac{H_i^2}{n}\right]$$

Here again the d.f., $4(n\text{-}1)$, were reduced by one, because of the missing valu-

The advantage for TDN substitution was compared to NE substitution averaged over both basal rations *(E)* and its standard error *(s_E)* are

$$\frac{-H_1 + H_2 - H_3 + H_4}{8n} \text{ and } \sqrt{\frac{3s_Q^2}{8n}},$$

respectively. The advantages in the 100% alfalfa ration and in the 80% alfalfa ration are

$$e(100) = \frac{-H_1 + H_2}{4n} \text{ and } e(80) = \frac{-H_3 + H_4}{4n}$$

both with standard error $s_E\sqrt{2}$. The additional advantage that TDN substitution in the 100% alfalfa ration has over TDN substitution in the 80% alfalfa ration is

$$d(100-80) = \frac{-H_1 + H_2 + H_3 - H_4}{4n}$$

with standard error $2s_E$.

Comparison of alfalfa with corn substituted on either the TDN or the NE basis.

When corn is substituted on the TDN basis, the advantage over the basal rations are

$$D + \frac{1}{2}E, d(100) + \frac{1}{2}e(100) \text{ and } d(80) + \frac{1}{2}e(80),$$

respectively, for the two basals combined, for the 100% alfalfa ration and for the 80% alfalfa ration. Similarly, when corn is substituted on the NE basis the advantages over the basal rations are

$$D - \frac{1}{2}E, d(100) - \frac{1}{2}e(100), \text{ and } d\,(80) - \frac{1}{2}e(80)$$

The contrasts that involve both basals have a standard error

$$s = \sqrt{\frac{35s_P^2 + 18s_Q^2}{192n}}$$

and those involving a single basal have a standard error $s\sqrt{2}$.

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RATE OF ABSORPTION OF CAROTENE AND OF VITAMIN A FROM THE ALIMENTARY TRACT OF DAIRY CALVES.[1] II. EFFECTS OF METHODS OF DISPERSION AND OF ADMINISTRATION

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SUMMARY

Different dispersions and methods of administration of vitamin A–active supplements on their apparent absorption by calves were studied. Vitamin A in an oily menstruum, an homogenized emulsion, and an aqueous dispersion—and carotenoids—in an oily solution and an emulsion—were mixed in a filled milk and fed via nipple. Vitamin A supplements, as an oily medium and as an aqueous dispersion, also in milk, were administered by a stomach tube. The rate of administration was 1,000 I.U/lb body weight. Changes in concentrations of vitamin A and of carotenoids in blood plasma, collected at 3-hr. intervals during a 15-hr. period after feeding the supplements, were the criteria of absorption. Concentrations of vitamin A in blood plasma increased and subsequently decreased more rapidly than did those of carotenoids. Emulsifying oily supplements in milk by homogenization significantly enhanced the apparent absorption of carotenoids but did not improve markedly the absorption of vitamin A. Aqueous dispersion of vitamin A effected more rapid passage into the blood and a higher maximum level than did either the emulsion or the oily solution. Ingestion of vitamin A by nipple resulted in more rapid absorption and higher concentrations in the plasma than did administration by stomach tube.

The frequent occurrence of vitamin A deficiency in cattle is the primary basis for continued exploration of factors affecting efficiency of utilization of this vitamin and its precursor. Among the many conditions that may influence the physiological economy of utilization are forms of dispersion *(4, 12)* and methods of administration *(7, 8)*.

The advent of dispersing agents has stimulated investigations of the effects of these preparations on the absorption of vitamin A and of carotenoids. Extensive work with simple-stomached animals has revealed that emulsification and aqueous dispersions enhance absorption of vitamin A–active compounds *(4, 12)*. Results from similar studies with cattle, though few, generally are concordant with observations on other species *(3, 7, 8, 9)*.

In view of the unique anatomical and functional characteristics of the ruminant stomach, the rate of passage of substances along the alimentary tract and the extent of exposure to biochemical agents may be affected by the physical state of the material and the methods of administration. Apparently, when vitamin A–active media pass directly into the abomasum, the rate of absorption is faster and the concentrations in the blood are greater than when the supplements enter the rumeno-reticular cavity *(7, 8)*.

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The need for further elucidation of the apparent absorption responses of calves to various forms of dispersions of vitamin A and of carotenoids, and to different methods of administering them, constituted the bases for the study presented herein.

EXPERIMENTAL PROCEDURE

Design. The principal variables in the investigation were, *(a)* different vitamin A–active supplements incorporated in a filled-milk diet, *(b)* methods of dispersing these supplements in the milk, and *(c)* the manner of administering vitamin A–supplemented milk to calves. Effects of these treatments on apparent absorption of carotenoids and of vitamin A were investigated in two primary series of trials, I and II, and in a secondary trial, which was a check test. In Series I and in the check tests, 12 calves of the Holstein breed were used, and in Series II, eight calves from Series I were continued. Each animal used in the respective series of trials was subjected to the treatments indicated (Table 1). The basic design employed in determining the sequential order of treatments within each series of trials was a Latin square.

TABLE 1

Methods of dispersing various concentrates of vitamin A and of carotenoids in filled milk and procedures in administering the supplemented milk to experimental calves

Series of trials	Vitamin A–active supplements			Method of incorporating supplements in milk	Mode of administering milk to calves[f]
	Substance	Medium	Potency[e] *(IU/g)*		
I	Vitamin A[a]	Fish liver oil	30,000	Homogenization Hand-stirring	Nipple feeder Nipple feeder
	Carotenoid[b]	Cottonseed oil	50,000	Homogenization Hand-stirring	Nipple feeder Nipple feeder
Basal-diet check		No supplement			Nipple feeder
II	Vitamin A[c]	Oil concentrate	100,000	Hand-stirring	Nipple feeder Stomach tube
		Aqueous dispersion[d]	12,500	Hand-stirring	Nipple feeder Stomach tube

[a] Supplied by the Borden Co., New York, through the courtesy of L. T. Wilson.
[b] Primarily β-carotene, purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.
[c] Supplied by Distillation Products, Inc., Rochester, N. Y., through the courtesy of P. L. Harris.
[d] Distilled natural ester concentrate emulsified by Tween 80 (polyoxyethylene sorbitan monoöleate) in the ratio of seven parts of dispersing agent to one part of oil.
[e] Listed values.
[f] Standard rate of administration was 1,000 I.U/lb of body wt.

Test diets. The basal diet, to which the respective vitamin A–active supplements (Table 1) were added, was a filled milk containing 10% nonfat milk solids, 3% hydrogenated soybean oil, and 87% water. The oil and the milk solids were dispersed in warm water, approximately 38° C., by thorough mixing followed immediately by homogenizing at 3,000 p.s.i. The respective vitamin A and carotenoid carriers (Table 1) were incorporated in the filled milk either *(a)* by adding the supplement during the mixing of the basal ingredients and

subsequently dispersing in the homogenization process, or *(b)* by adding it after homogenization and later dispersing by hand-stirring. To maintain a uniform distribution of this unhomogenized supplement in the milk, the mixture was agitated continuously as it was administered to the calf.

Feeding and management. During the three days immediately following the birth of each calf, it consumed colostrum directly from its dam. Thereafter, except when a test diet was given, fresh whole milk was fed routinely from a nipple pail. The rate of feeding was 10 lb. milk per cwt. of calf per day. In addition to the whole milk, each calf received daily a maximum of 0.5 lb. of a simple concentrate mixture fortified with minerals and vitamin D.

When the various treatments outlined (Table 1) were imposed on the calves, a regular morning feeding of whole milk was replaced with an equal quantity of one of the test diets (filled milk plus one of the supplements studied). No other feed was consumed during the subsequent 15 hr.

The test diets were administered, as previously described *(8)*, either from a nipple-feeder or by a stomach tube. Presumably, when the former method was used, nearly all the milk passed directly into the abomasum *(6, 15)*; whereas, when the latter was employed, passage was directly into the rumeno-reticular cavity *(8)*. Thus, to avoid flooding of these fore compartments of the stomach and consequent spillage into the abomasum, the quantity of filled milk used as a vehicle for the supplements (Table 1) was only one-third to one-half the total feed allowance when the stomach-tube administration was used. The remainder of the filled-milk allotment was given via a nipple feeder.

Trials of Series I were initiated when the calves were two weeks of age and were repeated at weekly intervals thereafter, until each calf had been subjected to treatments listed for this series (Table 1); whereas, trials of Series II were initiated when the calves were about 8 wk. of age and were continued through the treatment sequence at biweekly intervals. The special check trial, designed to ascertain responses to the basal diet per se, was conducted during the interim between Series I and II.

Sampling and analyzing blood. The criteria of responses to the treatments listed (Table 1) were carotenoid and vitamin A patterns of absorption, as reflected in diurnal changes in concentrations of these constituents in blood plasma of the calves. Blood samples were collected from the jugular vein of the calves immediately preceding the administration of the test diets and at 3-hr. intervals thereafter until the 15th hr. During the initial stages of this investigation, concentrations of carotenoids and of vitamin A in the samples of blood plasma were determined by a procedure described by Squibb *et al. (14)*; subsequently, however, the method was modified to include saponification *(1)*.

RESULTS

Series I. *Effect of dispersion procedure on concentrations of vitamin A and of carotenoids in blood plasma.* Diurnal patterns resulting from administrations of the respective vitamin A–active supplements, dispersed by hand-stirring

and by homogenization, are shown (in the first two sections of Figure 1). Mean values of vitamin A and of carotenoids in the samples of plasma from blood collected immediately prior to administrations of test diets were used as the reference base level, 0. The curves, therefore, represent changes from this pre-treatment value.

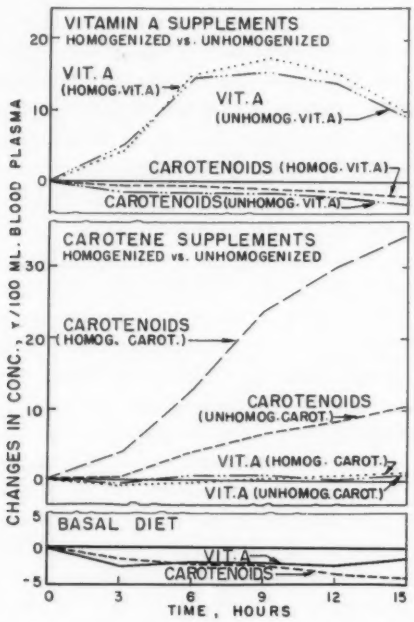

FIG. 1. Comparative diurnal changes in mean concentrations of vitamin A and of carotenoids in the blood plasma of calves receiving oily spplements, dispersed in a basal filled milk by homogenization and by hand-stirring.

The manner of dispersing the vitamin A supplement in the diet effected no pronounced difference in apparent absorption patterns in calves (as is revealed in the top section of Figure 1). Concentrations of plasma vitamin A increased to a peak between the 6th and the 12th hr., and declined subsequently. Levels of carotenoids in the same series of samples of blood plasma were not affected significantly by the form of vitamin A dispersion. Trends, however, during the 15-hr. period, were slightly downward.

In contrast to the vitamin-A curves, response patterns (middle section, Figure 1) resulting from feeding of carotenoids dispersed by the two methods were markedly different. Within the period of observation, the rates of increases and the magnitude of concentrations of carotenoids in blood plasma were greater from the homogenized supplement than from that which was hand-mixed. An analysis of variance of linear components, which were from a combination

of all values obtained from the sequential set of six samples collected during the 15-hr. period, indicated a significant difference ($P < 0.01$) in patterns of the carotenoid curves. Increases in plasma vitamin A concentrations, corresponding to the elevation in carotenoids, were only slight and were similar for the two forms of dispersions.

A comparison of the diurnal curves for vitamin A and for carotenoids indicates that the rate of increase to a crest was more rapid for the former than for the latter.

Effect of the basal diet on concentrations of vitamin A and of carotenoids in blood plasma. During the 15 hr. after the unsupplemented basal filled milk was fed, mean levels of carotenoids and of vitamin A in blood plasma of the calves decreased 3.7 γ/100 ml. and 1.6 γ/100 ml., respectively (bottom section, Figure 1). These reductions, evaluated by analysis of variance, were significant at the $P < 0.01$ level. Because values for samples of plasma collected immediately prior to administrations of test diets were used as reference bases in trials of Series I, the real magnitude of increases effected by supplements probably was greater than apparent values indicated.

Series II. *Effect of form of dispersion of vitamin A and of method of administration on concentrations of vitamin A and of carotenoids in blood plasma.* In Figure 2, curves showing means of observed concentrations, instead of differences from pretreatment values, as presented in Series I, indicate that the aqueous dispersion effected a more rapid increase of vitamin A in blood plasma and a higher concentration than did the oily medium. Intake via nipple also

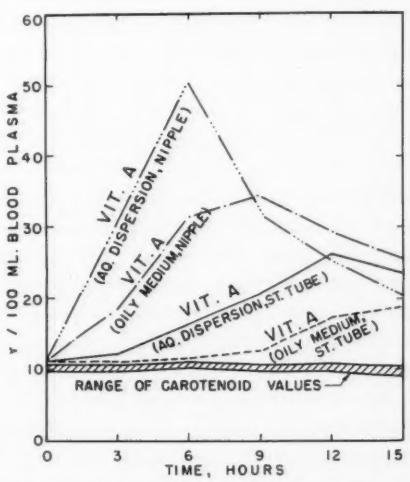

FIG. 2. Diurnal changes in the mean concentrations of vitamin A and of carotenoids in the blood plasma of calves that received vitamin A supplements, an aqueous dispersion and an oily mentruum, mixed in a basal filled milk, and administered by a nipple feeder and by a stomach tube, respectively.

resulted in a more rapid rate of increase and a higher level, within the 15-hr. period, than did administration by stomach tube. Patterns of response from administration of the two kinds of vitamin A supplements (oil concentrate and aqueous dispersion) by the two different methods (nipple feeder and stomach tube) indicate that rates of increases and maxima of concentrations are related positively. Furthermore, curves cresting within the 15-hr. period indicate that the higher the maximum level the more precipitous was the subsequent decline. The duration of observation, however, was too brief to reveal the general pattern resulting from giving the oily menstruum by stomach tube. The crest for the aqueous dispersion given from a nipple feeder occurred between 3 and 9 hr., which increase was more rapid than noted for vitamin A in oil administered similarly.

The maxima of the plasma vitamin A increases among the calves covered a wide range in the sequence of trials in Series II, but the relative order of response by individuals, as each was changed from one treatment to another, was strikingly uniform. The data revealed no apparent effects of either carry-over or age on responses.

Concentrations of carotenoids in the blood plasma were remarkably consistent throughout this series of trials. The spectrum of values (Figure 2) for the 15-hr. period apparently was not markedly affected by the vitamin A absorbed, even though the terminal level was slightly lower than the initial.

DISCUSSION

Results from the series of trials with calves, in accordance with findings reported for other species *(4, 12)*, indicate, for the most part, a close relationship between the forms of dispersions of carotenoids and of vitamin A in the diet, and the diurnal changes in concentrations of these constituents in blood plasma. Though it is recognized that the blood picture does not necessarily reflect accurately the efficiency of absorption *(7, 12)*, the response patterns are regarded as an acceptable index *(12)* in most instances.

The finding that emulsification of the carotenoid supplement by homogenization markedly enhanced the passage of this substance into the blood stream of calves *(16)* has been substantiated *(3, 9)*. Kon *et al. (9)* also found that when calves received carotene in an emulsion (homogenized), the resulting concentrations in the blood were as high as those from the consumption of an aqueous dispersion of carotene, and were maintained at the high level longer. Thus, state of dispersion of the carotenoid vehicle evidently plays an important role in absorption responses by calves as well as by other species.

Even though emulsification may not be so essential for absorption of vitamin A as it is for the epithelial passage of carotenoids *(4)*, the failure of the homogenized vitamin A concentrate to augment the elevation of vitamin A in plasma of calves significantly above the level resulting from the consumption of an oily solution, was contrary to expectations and discordant with findings from investigations with rats *(10)*. If the interpretation of the role of particle size in intesti-

nal absorption *(5)* is applicable to the utilization of vitamin A, the foregoing apparent discrepancy may be attributed to the failure of the homogenization pressure of 3,000 p.s.i. to reduce the particle size of the oily vitamin A vehicle sufficiently to be reflected prominently in the vitamin A concentrations in the blood. Further investigation, however, is necessary to explain decisively the results obtained with calves.

Differences in the diurnal patterns of vitamin A and of carotenoids, the former increasing to maxima more rapidly than the latter, and subsequently decreasing, are in accord with previous observations *(8)*. Vitamin A apparently not only is absorbed from the alimentary tract more readily than are carotenoids *(4)*, but also is removed from circulation more rapidly *(9)*.

The effectiveness of aqueous dispersions in accelerating the rate of transition of vitamin A to the blood of calves and in raising the peak of concentration is in harmony with observations on other species *(4, 12)*. The findings with calves *(16)* have been substantiated by Kon *et al. (9)* and supported by Jacobson *et al. (7)*. Sobel *et al. (13)* also found that an oily vitamin A concentrate dispersed in a sodium chloride solution by sorethytan laurate, and administered by stomach tube to lactating cows, effected a slightly higher concentration of vitamin A in the blood and a many-fold higher level in milk than did giving the same amount of vitamin A dissolved in corn oil.

Response patterns from different methods of administration (Series II) substantiate previous findings *(8)*, in which vitamin A supplements fed by nipple effected a more rapid increase in the concentrations of vitamin A in blood plasma and apparently higher maxima than did similar supplements administered by stomach tube. Among the many factors that may be involved, the most probable *(8)* included differences in pregastric exposure and in rate of alimentary passage, thereby possibly effecting a difference in losses of vitamin A potency and in efficiency of absorption. Obviously, explorations of a more definitive type are necessary to resolve the physiological complexity relating to the foregoing responses.

Unfortunately, the 15-hr. sampling period employed was too brief to characterize the entire pattern of changes resulting from the two methods of administration. Previous observations *(8)*, however, indicate that the durations of apparent absorption, pre- and post-maxima, were longer from the administration by the stomach tube than by the nipple feeder. Thus, in the total utilization of vitamin A initially deposited in the rumeno-reticular cavity, the efficiency from slow passage and uptake may be greater than the diurnal picture would indicate *(7)*.

The slight depression of levels of carotenoids in blood plasma following the absorption of vitamin A was not any greater than the decreases noted when the basal diet was fed. Also, the minor increase of vitamin A following uptake of carotenoids was statistically insignificant. Though the changes were in the direction anticipated, they were less than expected. These responses might have been amplified if the base levels of carotenoids in the blood had been higher when

vitamin A was administered, and if the vitamin A had been lower when the carotenoids were given. Kon *et al. (9)* found that within 4 hr. after the administration of carotene to calves low in vitamin A, increases in both alcohol and ester forms of this vitamin occurred. This finding is not in accord with that reported by Crowley and Allen *(3)*. Factors affecting interrelationships of vitamin A and carotenoids in the blood of calves receiving massive doses of either of these substances remain somewhat obscure.

Inasmuch as the observed levels of vitamin A and of carotenoids in the blood plasma represent only the state at the time of sampling, interpretations of the physiological significance of the response patterns in terms of net utilization are challengeable. However, recent investigations by Jacobson *et al. (7)* showed that when vitamin A in an aqueous dispersion was given to calves, not only absorption but also storage was greater than when the same unitage of vitamin A was administered in an oily solution. Since there is some doubt about the ability of cattle to convert injected carotenoids to vitamin A *(2, 9)*, the nutritional advantage from accelerating absorption of the precursor may not be so great as the gains from rapid passage of vitamin A. Kon *et al. (9)* found that when aqueous dispersions of carotene were given orally to calves ". . . carotene and vitamin A appeared in the blood more rapidly and in greater concentration, and much more carotene was deposited in the liver . . ." than when carotene in oily solution was administered, though the vitamin A stores were the same.

The findings reported herein indicate the potential importance of states of dispersions and of methods of administration of preparations of vitamin A and of carotenoids in the physiological economy of their utilization. These observations have possible therapeutic as well as prophylactic applications in calf husbandry. Utilization failures associated with impairment of absorption of carotene *(9)* or of vitamin A *(11)* in diarrhetic calves perhaps may be overcome partially through the administration of large amounts of aqueous dispersions of vitamin A. Acceptance of vitamin A–active aqueous dispersions for use in a normal calf regimen depends not only on improved efficiency of absorption but also on the cost of the preparations and their stability in storage.

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AN EVALUATION OF THE CHROMIC OXIDE METHOD FOR DETERMINING DIGESTIBILITY

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SUMMARY

Eight lactating dairy cows were used in a ten-day digestion trial, in which the conventional total collection method of determining digestibility was compared with the grab-sampling technique using chromic oxide as an indicator. Variations in the excretion of chromic oxide and crude fiber at 2-hr. intervals over a 24-hr. period were studied. Statistical analysis showed no significant differences between the two methods of determining the digestibility of the rations. Analyses of samples from the total fecal composites for the first six days of the ten-day trial revealed coefficients of digestibility comparable to those obtained for the entire period. Considerable variations were found in the chromic oxide content of the feces samples taken at various hours of the day, regardless of whether chromic oxide was administered once or twice daily. These variations make it difficult to select a sampling period which will give approximately 100% recovery for all cows for a given day; however, these data indicated this can be overcome in digestion studies by sampling the feces for a ten-day period. The hourly variations in the excretion of chromic oxide were not associated with the excretion of crude fiber.

Nutritionists are still confronted with the problem of measuring the feeding value of various rations and forages. For the past few years, considerable attention has been given to indicator methods, for determining the digestibility and intake of forages. Compared to the conventional methods for determining digestibility, indicator methods show great advantages, in saving time, labor, and equipment.

Hamilton *et al. (5)* and Crampton *et al. (3)*, using sheep, found the estimated coefficients of digestibility, using chromic oxide, to be comparable to those obtained from total collections. The former workers found that the length of the sampling period should be three or more days in length, in order to reduce sampling errors. The latter workers reported data which would indicate that the type of ration fed influences the results obtained from the chromic oxide method. When hay alone was fed, poor results were obtained, and upon the addition of grain to the ration the chromic oxide method checked more closely with the conventional method. This difference was explained on the basis of recovery of the chromic oxide, which was found to be only from 85 to 87% three days after chromic oxide administration had stopped, for sheep receiving the hay ration, as compared to from 98 to 99% for the same animals receiving a hay plus grain ration. This finding would indicate that the crude fiber level in the ration might conceivably affect the excretion of chromic oxide.

Kane *et al. (8, 9)* obtained good results in estimating the digestibility of alfalfa hay and silage fed to dairy cows, using the lignin ratio and chromic

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oxide method, when the fecal samples were removed from the total fecal collection. However, these results could be misleading, since sampling errors for both chromic oxide and the various nutrients would be eliminated. No advantage would be gained by using the chromic oxide method if total collections of feces had to be made in order to obtain a representative sample with respect to chromic oxide and the different nutrients. From a practical standpoint, the accuracy of the chromic oxide method can be determined only by grab sampling at hours of the day when the chromic oxide content of the feces approximates 100% recovery.

Some workers have been hesitant about accepting the chromic oxide method, because of the unknown cause of diurnal variations in its excretion, and the inability quantitatively to recover the amount ingested. Kane *et al. (10)* found highly significant differences between the levels of chromic oxide in A.M. and P.M. fecal samples. The highest concentration of chromic oxide in the feces was found near 9 A.M., followed by a gradual decline until 9 P.M. These variations in the excretion of chromic oxide were attributed to unknown internal biological laws.

Hardison *et al. (6)*, working with steers, and Lancaster *et al. (11)* and Smith *et al. (13)* with cows, found wide variations in the excretion of chromic oxide when the animals were grazing. Hardison *et al.* reported that steers hand-fed pasture forage exhibited an excretion pattern of chromic oxide similar to barn-fed cows *(6)* but different from grazing animals. This difference in excretion curves of grazing and hand-fed animals was attributed to time of feed intake. Smith found that the number of times (once or twice daily) or the mode of administration (capsule or in the feed) of chromic oxide had little or no effect upon the degree of accuracy of estimating fecal output of cows on pasture. Recently, Kameoka *et al. (7)* reported from a study with sheep that the kind of feed or the time of feed ingestion had no effect upon the diurnal variations in the excretion of chromic oxide. It was concluded that the daily variation in chromic oxide excretion was a normal occurrence amounting to about 10%.

The objectives of the present study were: *(a)* to compare the chromic oxide method with total collections of feces for determining digestibility; *(b)* to compare a six-day total collection period with a ten-day period; *(c)* to study the effects of the number of administrations of chromic oxide daily upon the hourly variations of this inert material; and *(d)* to check the possible relationship of the hourly excretion of chromic oxide to crude fiber.

EXPERIMENTAL PROCEDURE

I. Digestion studies. Eight lactating dairy cows, receiving four different rations, were selected on the basis of dry matter intake, milk production, and body weight. The grouping of the cows, along with the four different rations fed, is given (Table 1). During a ten-day preliminary period, the cows were given 5 g. of chromic oxide in a gelatin capsule twice daily, at 9 A.M. and

TABLE 1

Grouping of cows and rations fed during the digestion trial

Ration	Cow No.	Av. daily intake			Daily dry matter intake
		Grain[a]	Hay[b]	Silage[c]	
		(lb.)			
I	1427	12.0	.....	72.0	33.2
	1471	8.0	.....	55.0	24.4
II	1435	14.0	.....	58.0	30.3
	1446	12.0	.....	68.0	31.4
III	1478	16.0	13.7	34.0	37.8
	1249	18.0	13.0	26.0	36.4
IV	1392	14.0	14.2	30.0	35.1
	1469	14.0	13.0	22.0	31.4

[a] The grain for Ration I was ground shelled corn—for Ration II, ground shelled corn + 3.5% urea—for Ration III, ground shelled corn, and for Ration IV, regular herd mix (15% crude protein).

[b] Good alfalfa hay.

[c] Corn silage.

9 P.M. These hours were selected only for the sake of convenience. During the ten-day collection period, the cows remained in their respective stanchions and total fecal collections were made manually. The fecal grab samples were taken at 6 A.M. and 4 P.M. and composited over the ten-day period. Dry matter content of the daily fecal output was determined, for use in making the final composite. Portions of the daily fecal sample, for the first six days of the ten-day trial, were composited on a dry matter basis and analyzed separately, to determine the digestibility of the rations as compared to the ten-day total.

II. Hourly variations in the excretion of chromic oxide within a 24-hr. period. Three days following the digestion trial, and while the cows were still being given 5 g. of chromic oxide twice daily, fecal grab samples were taken at 8 A.M. and at 2-hr. intervals thereafter for a 24-hr. period. These samples were dried in a forced-air oven, and analyzed for dry matter and chromic oxide.

Since the excretion pattern of chromic oxide by dairy cows *(10)* and steers *(6)* has been shown to follow the same trend if similar rations are fed, another group of eight cows was selected for the study of the effects of administering 10 g. of chromic oxide once daily (9 P.M.) upon the hourly variations in excretion of this inert substance and the relationship of the excretion of crude fiber and chromic oxide. The procedure for this trial was similar to that previously given for the first trial. During the course of the study one cow, receiving Ration I, was removed because of a digestive disturbance which resulted in reduced feed consumption.

The chromic oxide content of the feces was determined by the method of Gehrke and Baker *(4)*. This method proved to be very rapid and to yield excellent recoveries. Other analyses were made according to accepted A.O.A.C. methods *(1)*.

RESULTS AND DISCUSSION

I. Digestion studies—Chromic oxide versus total collection for determining digestibility. The data for the digestibility of dry matter as determined by the two methods are shown (Table 2), along with the data obtained from the six-day total collection. Two comparisons can be made from these data: *(a)* the accuracy of the chromic oxide method in determining the digestibility of dry matter, as compared to the conventional method; and *(b)* the effect of the length of the sampling period, six days versus ten days, upon the digestion coefficient of dry matter.

TABLE 2

Coefficients of apparent digestibility of dry matter

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	65.4	63.6	63.7
	1471	71.1	73.6	72.5
II	1435	64.3	64.7	62.3
	1446	67.3	67.4	67.7
III	1478	70.9	67.0	72.4
	1249	67.4	69.3	70.0
IV	1392	69.2	68.4	67.2
	1469	69.8	65.6	73.3
	Mean	68.2	67.5	68.6

[a] Grab samples were taken for ten consecutive days.

An analysis of variance of the data, as outlined by Snedecor *(14)*, showed no significant difference between digestion coefficients obtained using the chromic oxide method and those for the ten-day total collection.

No significant differences were found between data of the six-day and the ten-day total collection periods. This is in agreement with other findings at this station *(2)*. However, one must keep in mind that the length of the preliminary period would be a major factor in determining the length of time to collect feces, in order to eliminate errors in sampling. The cows used in this study had been consuming their respective rations for several weeks and were maintained on a constant feed intake during the entire trial.

The coefficients of digestibility for the crude protein and crude fiber are presented (Tables 3 and 4, respectively). In determining the digestibility of protein and crude fiber, more variation existed between methods than in the case of dry matter digestibility. These differences, however, were not statistically significant. Except for ash, the methods studied gave coefficients of digestibility for nitrogen-free extract and ether extract with about the same degree of accuracy as was found for crude protein and crude fiber. Ash was somewhat more variable, as is normally the case.

From these results, the chromic oxide method was found to give coefficients of digestibility comparable to those obtained by the conventional method.

TABLE 3
Coefficients of apparent digestibility of crude protein

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	60.5	58.1	59.9
	1471	59.6	60.7	59.6
II	1435	65.7	64.5	64.1
	1446	67.6	65.9	68.1
III	1478	66.7	64.3	67.5
	1249	74.2	72.4	73.9
IV	1392	64.7	64.8	66.5
	1469	76.4	70.4	77.5
	Mean	66.9	65.1	67.1

[a] Grab samples were taken for ten consecutive days.

II. Hourly variation in the excretion of chromic oxide. Chromic oxide administered once daily. Smith *et al. (13)* found that the fecal output of grazing cows could be estimated with about the same degree of accuracy, whether the chromic oxide was administered once daily, or twice. Lassiter *et al. (12)*, working with steers in a standard digestion trial, found less hourly variations in the excretion of chromic oxide when chromic oxide was administered twice, as compared to once, daily. If some of the hourly variation in the excretion of this indicator could be eliminated by the number of times of administration, the error of sampling could likewise be reduced.

A graphic illustration of the variation in the excretion of chromic oxide by seven cows for a period of 24 hr. is contained in Figure 1. The data are presented as per cent of the chromic oxide in the grab sample, compared to the mean chromic oxide content of all the grab samples taken for each cow during the 24-hr. period. The grab samples were removed from the rectum of the cow at the time specified. Six of the seven cows showed the highest level of fecal chromic oxide between the hours of 12 NOON and 6 P.M., and the lowest content

TABLE 4
Coefficients of apparent digestibility of crude fiber

Ration	Cow No.	Method		
		10-day total collection	Cr_2O_3[a]	6-day total collection
		(%)		
I	1427	62.3	60.2	62.3
	1471	66.5	68.3	68.9
II	1435	53.6	61.6	60.3
	1446	57.7	60.0	64.3
III	1478	54.8	56.3	59.6
	1249	51.4	49.9	53.8
IV	1392	45.2	51.8	54.4
	1469	48.2	45.5	57.6
	Mean	55.0	56.7	60.1

[a] Grab samples were taken for ten consecutive days.

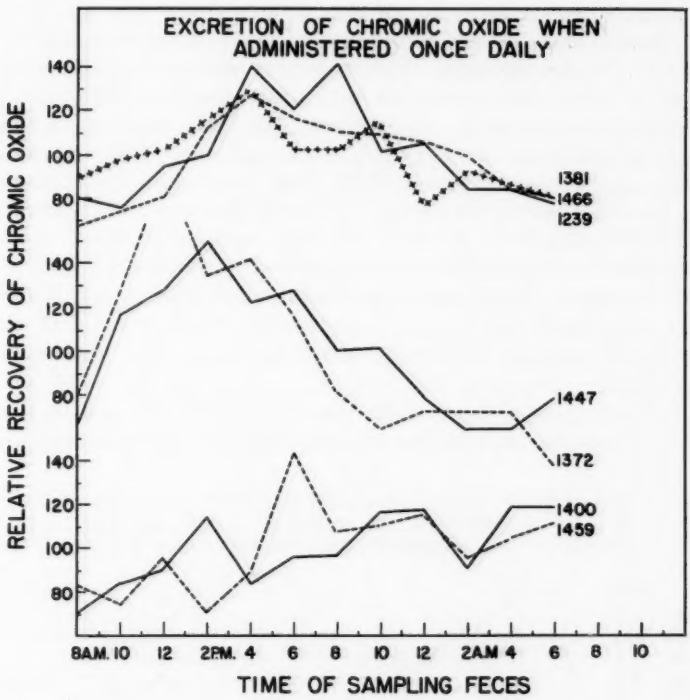

FIG. 1.

between the hours of 2 A.M. and 8 A.M. Five of the seven cows showed similar trends in the excretion of chromic oxide. The two remaining cows (No. 1400 and No. 1459) exhibited excretion curves different from the other cows, but similar to each other, in that the concentration of chromic oxide in the feces increased, instead of decreasing, after 4 P.M. Attempts to correlate the excretion curves of chromic oxide with dry matter intake and rations fed, failed to explain the cause of these variations. The average range in the rate of recovery of the chromic oxide from the feces was from 76 to 119%. Cow No. 1372 showed extreme variation (from 45 to 188%), but the excretion curve for this cow followed the same general trend as was found for cows showing less variation. The recommended time to grab sample *(6)*, to obtain samples which represented more nearly the mean chromic oxide content for a 24-hr. period, was found to be near 4 P.M. and 6 A.M. Using these two sampling periods, the mean per cent recovery of the chromic oxide for the seven cows was 102%, with a standard deviation of $\pm 4.3\%$.

Based on the findings of Crampton *et al. (3)*, in which poor recoveries of chromic oxide from the feces were obtained from feeding sheep an all-roughage

ration, an attempt was made to correlate hourly crude fiber excretions with those of chromic oxide. Crude fiber was determined on the grab samples taken for chromic oxide determination. The crude fiber content of the feces displayed a different excretion pattern and was less variable than that found for chromic oxide. Two of the seven cows (No. 1447 and No. 1239) excreted crude fiber at a uniform rate over the 24-hr. period. The mean crude fiber contents of the fecal grab samples were 28.0 and 30.9%, with a standard deviation of ± 1.6 and $\pm 1.4\%$, respectively. In general, the crude fiber excretion pattern agreed with the excretion pattern of lignin by cows, reported by Kane *et al. (10)*. This would be expected, because a large portion of the crude fiber fraction is composed of lignin. These results demonstrate that crude fiber and chromic oxide are excreted independently of each other, and that the diurnal variations in the excretion of chromic oxide are not associated with the excretion of crude fiber.

Administration of chromic oxide twice daily. The excretion of chromic oxide over a 24-hr. period when the chromic oxide was administered twice daily

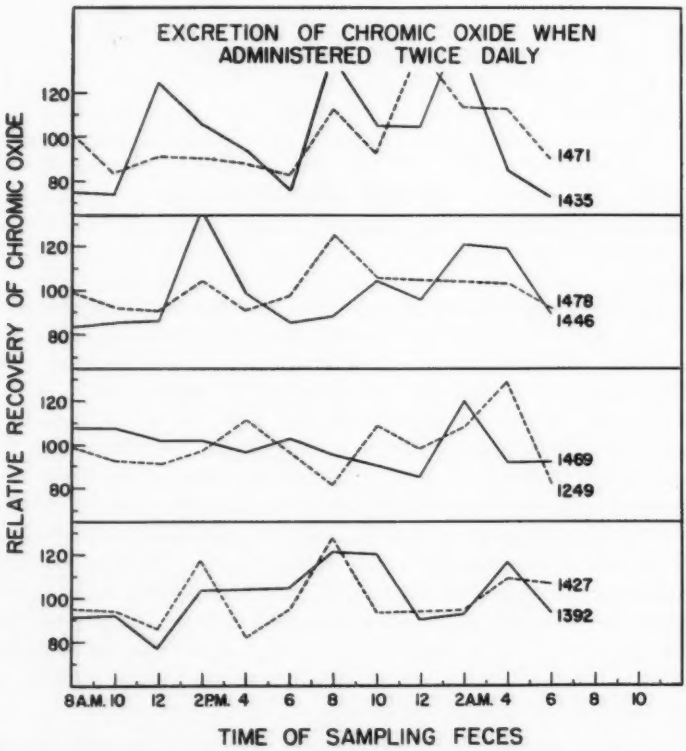

FIG. 2.

is shown (Figure 2). Data are presented as the per cent of the mean chromic oxide content of all fecal grab samples for each cow taken for the 24-hr. period. This enables one to make direct comparison of the excretion of chromic oxide in the feces of cows receiving different quantities of feed. Twice-per-day administration of chromic oxide appeared to lessen the variation in its excretion; however, considerable fluctuation still occurred. Even though less variation was noticed in hourly excretion rates of chromic oxide, the general excretion trend was erratic and would make it very difficult to devise a sampling plan in which the grab samples would give chromic oxide values near the daily mean value for all eight cows. Selection of the hours, 6 A.M. and 4 P.M., as previously used, results in an over-all mean recovery of 93% with a standard deviation of $\pm 4.1\%$. The range in per cent recovery for the eight cows, using these hours, was from 83 to 97%. Since these cows were used in the digestion studies in which the chromic oxide method gave satisfactory results, the data are indicative that there are day-to-day fluctuations in the excretion pattern of this indicator, which are overcome by sampling ten days.

ACKNOWLEDGMENTS

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USE OF SODIUM PROPIONATE IN THE PREVENTION OF KETOSIS IN DAIRY CATTLE[1]

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SUMMARY

In a study involving 100 high-producing cows, one-half served as controls and one-half was fed 0.25 lb. of sodium propionate daily for a period of 6 wk., starting at calving. Blood analyses and milk production showed an advantage for the propionate-fed cows in terms of higher blood sugar levels, lower blood ketones, and higher milk production. Greatest differences were observed at 3 wk. after calving, when they were highly significant in favor of the propionate-fed cows in all three measurements. At 7 wk. after calving, after the propionate had been discontinued, there were no significant differences between the two groups. During the 6-wk. feeding period, nine cows in the control group showed sufficient symptoms of ketosis to require treatment; whereas, the level of propionate was increased on two cows in the propionate-fed group because of an abnormal blood picture.

The economic losses to the dairyman resulting from ketosis are well known. A recent review by Shaw *(13)* gives a comprehensive report of the history and present status of research on the problem.

Several fairly satisfactory treatments for ketosis have been developed. The ones most commonly being used in this country include glucose injection *(8)*, injection of glucocorticoids *(14)*, or the oral administration of sodium propionate *(9)*, propylene glycol *(6)*, or lactates *(13)*. Although these treatments, either alone or in combination, are reasonably satisfactory, prevention of the condition would be more satisfactory if it could be accomplished in a practical and economical manner.

Many suggestions have been made for the prevention of ketosis, but lack of controlled experiments makes them difficult to evaluate. The most common feeding practices recommended for this purpose include the feeding of molasses *(8)*, cobalt *(17)*, or high-protein rations *(15)*. The experimental evidence available *(13)* casts doubt on the value of molasses as an aid in ketosis prevention. No convincing experimental evidence has been presented to show that extra cobalt or protein above the amounts called for in our feeding standards is beneficial from the standpoint of ketosis. There seems to be fair agreement that such things as not having cows excessively fat at calving time, bringing them up to full feed rapidly after calving, and feeding high-quality roughage, are helpful in preventing ketosis, but they are based primarily on field observations which are difficult to evaluate.

Of the treatments, only those given orally seem to be very well adapted to use for prevention. After the initial report *(9)* on the use orally of sodium

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propionate for the treatment of ketosis, it became obvious that this material might have some value in prevention. Other indications that it might be useful for this purpose came from observations that the introduction of propionic acid into the rumen of normal animals caused marked increases in blood sugar *(12)*, and that the level of propionic acid in the rumen was low at the time of ketosis *(2, 11)*.

The purpose of this study was to determine whether the feeding of sodium propionate for a period of 6 wk. after calving would prevent ketosis. The results of this study were reported upon earlier, in abbreviated form *(10)*.

EXPERIMENTAL PROCEDURE

The study covered a 3-yr. period and involved 100 cows (50 pairs) in three herds. Forty-four pairs of Holsteins, three pairs of Jerseys, and three pairs of Brown Swiss were used.

The cows within each herd were paired as equally as possible on the basis of previous production, history of ketosis, age, and time of calving. One member of the pair served as a control and the other, selected at random, was fed sodium propionate in the grain at a level of 0.25 lb. daily for a period of 6 wk., starting at calving.

In the case of 12 pairs, the propionate was measured out in a plastic cup for each cow at each feeding and mixed into the grain by hand. For the remaining pairs, the propionate was pelleted into part of the grain mix so that the final level was 2.5% of the special feed. By feeding 5 lb. of this special mix night and morning (10 lb. per day), an intake of 0.25 lb. of sodium propionate per cow per day was obtained. Any grain needed, in addition to the 10 lb., was made up of the regular grain mix without propionate. Within each herd all cows were fed the same roughage and grain mix and were handled normally. Grain was fed according to production.

Blood samples for sugar and ketone determinations were taken at 1, 3, 5, and 7 wk. after calving. The exact sampling date varied a maximum of two days from that scheduled, to permit twice-a-week sampling. Additional samples were taken if ketosis was suspected, and before and after treatment in the case of cows treated for ketosis. All blood samples were taken from the jugular vein. Methods for determining blood sugar and blood ketones were the same as those described in previous papers *(9, 12)*. Milk production was recorded on the day of sampling (in one herd, on the day before sampling). All cows were milked twice daily.

When complicating difficulties were encountered in one member of a pair, the pair was removed from the experiment. Metritis was the most common complication. Milk fever cases were not removed from the experiment, because they all recovered satisfactorily after treatment. Two of the experimental cows failed to eat the propionated feed well, so the pairs involved were removed from the experiment. Both of these cows had a history of being finicky eaters.

Ketosis was diagnosed only when all of the following symptoms were observed: *(1)* the cow was off feed, *(2)* production was down, *(3)* blood sugar was

low and blood ketones were high. Cows with ketosis were treated with sodium propionate. It was given by drench at a level of 0.50 lb. per day for as long as necessary, usually from five to ten days. All cows responded satisfactorily.

RESULTS

Table 1 gives a summary of the blood sugar, blood ketone, and milk production information on the 50 pairs of cows at the regular sampling intervals of 1, 3, 5, and 7 wk. after calving.

TABLE 1

Effect of feeding 0.25 lb. of sodium propionate daily for 6 wk. after calving, on blood sugar, blood ketones, and milk production (50 pairs of cows)

Time after calving	Control		Propionate-fed		Mean difference
	Mean	Range	Mean	Range	
1 Week					
Blood sugar *(mg.%)*	44.5	29.2-59.0	46.9	29.6-65.4	2.4
Blood ketones *(mg/%)*	11.0	1.0-35.0	8.9	2.6-19.9	2.1[a]
Milk production *(lb/day)*	51.2	28.1-70.2	54.4	34.6-73.0	3.2
3 Weeks					
Blood sugar *(mg%)*	42.8	25.0-63.6	48.0	24.6-59.2	5.2[b]
Blood ketones *(mg. %)*	15.7	3.5-46.5	9.9	3.8-23.9	5.8[b]
Milk-production *(lb/day)*	55.7	34.2-72.8	62.0	35.8-80.7	6.3[b]
5 Weeks					
Blood sugar *(mg. %)*	46.4	24.3-66.3	50.0	37.5-63.2	3.6[b]
Blood ketones *(mg. %)*	13.8	3.2-49.4	10.4	3.2-25.2	3.4[a]
Milk production *(lb/day)*	58.3	29.1-80.6	62.9	28.6-91.5	4.6[b]
6 Weeks — Propionate feeding discontinued					
7 Weeks					
Blood sugar *(mg. %)*	50.2	37.9-66.7	50.2	37.1-59.5	0
Blood ketones *(mg. %)*	10.5	4.2-32.4	8.5	3.5-26.7	2.0[a]
Milk production *(lb/day)*	59.1	33.6-83.5	60.0	18.1-93.0	0.9

[a] Differences significant ($P < 0.05$).

[b] Differences highly significant ($P < 0.01$).

It can be seen that the mean blood sugar level in the case of the control cows declined to a low point 3 wk. after calving and did not reach what are considered normal levels until 7 wk. after calving. Blood ketones showed the typical reciprocal relationship. In the case of the propionate-fed cows, the mean blood sugar levels were consistently higher and increased gradually, reaching normal levels at 5, rather than 7, wk. after calving. The mean blood ketone levels of the propionate-fed cows were consistently lower and reached a peak at 5, rather than 3, wk. after calving.

In the case of average milk production, the control cows showed a gradual increase in production, with maximum average production not being reached until 7 wk. after calving, when the blood sugar was normal and the blood ketones approached normal levels. The lower-producing cows appeared to reach their peak production earlier than the higher producers, which were more susceptible to ketosis. Average daily production of the propionate-fed cows was consistently higher, reaching a peak at 5 wk. and declining slightly at 7 wk. after the propionate was removed.

The mean differences between the control and propionate-fed cows in all three measurements were small at first, reached a maximum 3 wk. after calving, and essentially disappeared after the propionate was discontinued. In the case of blood sugar, the small difference in favor of the propionate-fed group 1 wk. after calving was not statistically significant. The differences increased, to become highly significant at 3 and 5 wk. after calving, then declined to zero at 7 wk. Blood ketones were significantly lower for the propionate-fed cows at 1, 5, and 7 wk. after calving, whereas at 3 wk. after calving the difference was highly significant. Differences in milk production were small and non-significant at 1 wk., became highly significant at 3 and 5 wk. after calving, and essentially disappeared after the propionate was discontinued.

The ranges in values (Table 1) show that the extremes were not too different in the two groups, except that blood ketones in the propionate-fed cows did not go to the extremely high levels observed in some of the control cows. Although the average blood sugar level of the propionate-fed cows was higher, the propionate feeding did not prevent a blood sugar drop in some of the cows.

Effect of the propionate feeding on the incidence of ketosis and the extent of the changes in blood sugar and blood ketones are shown (Table 2). There were nine cases of ketosis treated in the control group, whereas the level of propionate was increased to ⅜-lb. daily on two cows in the propionate-fed group. This was done because the blood picture was abnormal and milk production was down, although the cows were not off feed.

TABLE 2

Effect of feeding 0.25 lb. sodium propionate daily for 6 wk. after calving on the incidence of ketosis and the extent of changes in blood sugar and ketones (50 pairs of cows)

	Control	Propionate-Fed
Number of cases of ketosis treated	9	Level increased for 2 cows
Number of cows dropping below following levels blood sugar:		
40 mg/100 ml.	23	7
35 mg/100 ml.	14	2
30 mg/100 ml.	7	1
Number of cows increasing above following levels of blood ketones:		
20 mg/100 ml.	18	8
25 mg/100 ml.	12	1
30 mg/100 ml.	7	0
Mean minimum blood sugar *((mg/100 ml.)*[a]	39.1	43.8
Time of minimum sugar in days after calving	16.4	18.1
Mean maximum blood ketones *(mg/100 ml)*[a]	19.1	12.8
Time of maximum ketones in days after calving	22.4	21.6

[a] Differences highly significant ($P < 0.01$).

Table 2 also shows that the feeding of propionate reduced the number of cows showing an abnormal blood picture. Roughly 40% of the control cows showed at least a somewhat abnormal blood picture (below 40 mg. % sugar, above 20 mg. % ketones) some time during the first 6 wk. after calving. About half of these were not treated, because other symptoms were not serious enough.

The differences between the control and propionate-fed cows in minimum blood sugar and maximum blood ketone levels were both highly significant statistically in favor of the propionate-fed cows. The average times at which minimum sugar and maximum ketones were reached apparently were not influenced by the treatment.

Table 3 gives the blood sugar, blood ketones, and milk production of the cows developing ketosis and the borderline cases, at the various sampling intervals. All cows with blood sugar levels below 35 mg. % and blood ketones above 20 mg. %, at some time during the first 6 wk. after calving, were considered borderline. The reason they were not treated was that they were not reported to be off feed by the respective herdsmen.

It can be observed (Table 3) that all of the ketosis cases occurred between 1 and 5 wk. after calving. Most of these cases were slow in developing and could be predicted from the blood samples taken 2 wk. previously. The data on the two cows on which the propionate level was increased suggest that a level higher than ¼-lb. daily would be beneficial for certain cows.

The information on the borderline ketosis cases (Table 3) shows that cows can have a blood picture definitely indicating ketosis and still be eating and milking fairly well. Some of the borderline cows had a more abnormal blood picture than some of the treated cows. It is certainly likely, however, that these borderline cows would have responded to treatment with substantial increases in production. The highest producing cow in the whole study was the propionate-fed Cow 16H, with borderline ketosis, as shown in Table 3. She was milking over 80 lb. daily with a blood sugar level of less than 25 mg. % and was eating very well. She did, however, go over 90 lb. when the blood sugar returned to more normal levels.

Several of the propionate-fed cows that were apparently susceptible to ketosis dropped noticeably in production when the propionate was removed, but none developed ketosis.

No breakdown of the data by herd or by year is included. A statistical analysis of the blood sugar values showed that there were no significant differences due to herd or year. Services per conception were essentially the same for both treatments.

DISCUSSION

Results of this experiment appear to have both practical and theoretical significance. From a practical standpoint, it was shown that under the conditions of this experiment, it was possible to maintain higher blood sugar levels, lower blood ketones, and higher milk production, as well as to reduce the incidence of ketosis, by feeding sodium propionate. However, the fact that some of the propionate-fed cows showed a somewhat abnormal blood picture indicates that 0.25 lb. daily will not maintain a normal blood picture on all cows. Even at increased levels it is doubtful that it will prevent ketosis in all cows, because of the complicated nature of the condition. Certainly, its limitations under field conditions, where numerous complications are involved, should be recognized.

TABLE 3

Blood sugar, blood ketones, and milk production of cows developing ketosis and of borderline cases

Pair[a]	Weeks after calving: 1			3			5			7		
	Sugar	Ketones	Milk	Sugar	Ketones	Milk	Sugar	Ketones	Milk	Sugar	Ketones	Milk
	(mg. %)	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*	*(mg. %)*	*(mg. %)*	*(lb/day)*
Control cows developing ketosis												
1H	45.9	10.1	45.0	38.3	20.2	47.9	30.2	49.4	29.1[b]	49.0	17.4	52.4
9H	46.5	8.6	63.0	49.6	6.2	72.8*	50.7	5.5	80.6	38.3	16.0	63.7[c]
10H	50.0	4.9	50.4*	55.2	4.4	60.5	52.8	5.3	59.9	56.2	6.5	63.4[c]
27H	47.9	12.2	68.4	29.6	28.1	68.6[b]	37.5	32.9	72.7	43.4	18.0	83.5
28H	39.9	15.2	60.8	35.5	26.0	60.0*	46.5	14.4	68.2	52.8	6.9	65.5[c]
30H	36.9	17.5	31.4	25.7	29.3	40.2[b]	44.4	12.7	58.4	44.3	11.9	58.8
34B	40.3	22.5	42.7	28.1	46.5	37.7[b]	40.2	42.7	45.0	46.5	12.1	48.5
40H	31.6	35.0	59.7[b]	32.1	25.1	61.6	46.5	21.3	58.6	43.9	16.2	63.8
50H	37.7	25.8	54.8	26.9	42.7	46.5[b]	51.4	6.2	77.0	56.6	4.2	80.5
Propionate-fed cows for which level of propionate was increased												
30H	40.1	16.8	54.0	38.9	22.8	56.1	37.5	25.2	53.4[b]	50.4	5.7	64.7
50H	29.6	12.2	65.1[b]	45.0	5.9	68.2	56.2	3.4	82.5	56.4	4.2	79.2
Borderline[d] control cows not treated because not off feed												
4H	40.9	21.8	55.3	34.3	23.0	56.5	46.0	14.5	62.8	46.0	8.7	62.1
5H	33.5	13.9	60.1	31.1	19.9	64.3	37.1	30.8	60.9	37.9	19.1	70.1
14H	29.2	18.5	51.4	27.1	36.7	57.0	24.3	32.4	57.4	48.9	32.4	69.4
25H	36.9	8.9	63.0	25.0	37.0	59.0	34.5	30.1	74.0	42.8	22.6	77.2
37H	40.3	8.3	61.3	33.2	26.4	60.0	46.0	16.4	72.1	42.4	14.5	73.3
45H	47.9	6.3	57.9	33.1	16.4	67.5	28.8	20.1	54.2	55.7	8.3	62.0
Borderline[d] propionate-fed cow not treated because not off-feed												
16H	37.1	11.0	73.0	24.6	20.7	80.7	43.4	19.4	91.5	48.9	12.4	93.0

[a] H = Holstein, B = Brown Swiss.

[b] Treated for ketosis at time indicated.

[c] Treated for ketosis between sampling intervals at time indicated by *.

[d] All cows with blood sugar levels below 35 mg. per 100 ml., and blood ketones above 20 mg. per 100 ml., considered borderline.

In this experiment, ketosis was controlled in both groups of cows. In one group it was by a prevention schedule, in the other by treatment of the cases as they developed. Less propionate was used for the treatment schedule, because only 18% of the cows were treated and the amount used per cow was somewhat less. However, the propionate-fed cows did produce approximately 200 lb. of milk per cow more during the 6-wk. feeding period. This increased production appeared to be owing to the fact that they were able to maintain production closer to normal rather than any stimulation above normal.

Under field conditions, whether or not a preventive schedule of this type would be practical would appear to depend upon several factors, such as the incidence of ketosis, the level of production, the price of milk, and the cost of propionate. It would appear to have its greatest value when used on selected cows, in high-producing herds with a high incidence of ketosis. In addition to their use for strictly preventive purposes, other possible uses of feeds containing propionate are as a treatment for mild cases of ketosis while the cow is still eating, or as a follow-up treatment, to prevent relapses.

Other observations of practical importance, aside from the specific objective of the experiment, included the following: *(1)* Most of the high-producing cows passed through a borderline ketosis period during the first 6 wk. after calving. Feeding and management practices during this period are critical. Oral ketosis-control measures offer a convenient way of handling these borderline cases that are still eating fairly well. The high average values for blood ketones suggest that care should be exercised in the interpretation of the qualitative urine test for ketosis during this period. Not all cows with a positive urine test require treatment. *(2)* The time of occurrence of the ketosis cases and the general trend of blood sugar and blood ketone levels showed that the problem period was limited to approximately the first 2 mo. after calving. This suggests that difficulties outside this period would not likely be ketosis. *(3)* Ketosis cases which occurred were slow in developing. In most cases, there was a gradual increase in blood ketones and a gradual decline in blood sugar, milk production, and appetite. Usually, the blood picture showed abnormalities 2 wk. before the cows were actually treated. This indicates that when a uniform system of feeding and management is followed during this period, and when the cows are observed carefully, cows going off in feed and milk abruptly are apt to have something other than ketosis as the primary cause of their difficulty. Abrupt changes in feeding or management, on the other hand, could rapidly throw the borderline cases into ketosis.

This work adds additional support to the idea suggested by the use of sodium propionate for treatment: that it might be possible to control ketosis by nutritional means. The theoretical reasoning behind it involves the concept that propionate serves as a glucose precursor, which is more effective orally than glucose itself, because it escapes bacterial action in the rumen and is absorbed directly. The manner in which propionate may fit into the scheme of intermediary carbohydrate metabolism after absorption has been reviewed by several workers *(1, 5, 7)*. The other three carbon compounds effective orally apparently follow

either the same ultimate pathway and serve as glucose precursors or, as is the case with lactic acid under most normal circumstances, are converted in large part to propionic acid before absorption *(4, 16)*. The fact that such a small amount of propionate gave as much response as was observed is somewhat surprising. The increased milk production cannot be completely accounted for by the propionate consumed. Apparently, it was not only a matter of conversion of propionate to glucose to lactose, but an effect on the general well-being of the cow in terms of increased efficiency of intermediary metabolism and/or increased feed intake.

One interesting possibility suggested by this work is that by feeding certain feeds or combinations of feeds, or by the use of special inoculum, one might cause increased production of propionic acid in the rumen during this ketosis-susceptible period, thus accomplishing the same results without adding propionate to the feed. It has been demonstrated by several workers that the proportions of volatile fatty acids in the rumen may be changed by alterations in the diet *(3, 13)*. Work on this aspect of nutritional control of ketosis is under way.

ACKNOWLEDGMENT

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EFFECT OF FEEDING SODIUM PROPIONATE ON MILK AND FAT PRODUCTION, ROUGHAGE CONSUMPTION, BLOOD SUGAR, AND BLOOD KETONES OF DAIRY COWS[1]

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SUMMARY

A double-reversal experiment involving ten cows from the Cornell University Dairy Herd was used to detect the effect of the daily feeding of ½ lb. of sodium propionate on milk production, butterfat test, hay consumption, blood sugar and blood ketone levels. There were no significant differences ($P < 0.05$) in the milk production, hay consumption, blood sugar levels, blood ketone levels, or weight gains or losses attributed to the feeding of sodium propionate. There was a slight but nonsignificant fall in butterfat test.

In a supplemental experiment, two fistulated steers were fed propionate and control feed, and the fatty acid content of the rumen was determined at regular intervals after feeding. The feeding of the propionate feed resulted in higher levels of all the volatile fatty acids in the rumen for the first 3 hr. after feeding, particularly the propionic. From 3 to 9 hr. after feeding, the concentrations of rumen acids from the two feeds were quite similar. It was calculated that all of the administered propionate had disappeared in 7 hr., and most of it in 3 hr.

Sodium propionate has been shown to be effective in the treatment and prevention of ketosis. Schultz *(9)* found that the oral administration of ½ lb. of sodium propionate daily for from three to ten days to cows with uncomplicated ketosis was effective. Schultz *(10)* also found that the daily feeding of ¼ lb. of sodium propionate to cows for 6 wk. after calving reduced the incidence of ketosis. The propionate-fed cows showed higher blood sugar levels, lower blood ketone levels, and produced more milk for the first 6 wk. after calving than did the control cows.

Because of the stimulation in production observed when sodium propionate was fed or administered to cows with mild to severe ketosis, it was felt desirable to determine the effect of feeding it to normal cows. Information on this point was requested by those associated with official testing programs of the breed associations.

Tyznik and Allen *(11)* found that cows having a low butterfat test due to a high concentrate–low roughage diet showed a decreased percentage of acetic acid in the rumen, an increased percentage of propionic acid, and a normal percentage of butyric acid. When acetate was fed to these cows, the butterfat tests returned to normal. Miller and Allen *(7)* reported that the daily feeding of 1 lb. of sodium acetate to normal cows did not affect significantly the milk production, fat test, blood sugar or ketone values.

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[1] The data published in this paper are from a thesis presented by the senior author to the Graduate School, Cornell University, in partial fulfillment of the requirements for the degree of Master of Science, February, 1956.

This experiment was designed to determine whether the feeding of sodium propionate would affect the milk production, fat test, roughage consumption, blood sugar and ketone levels of normal cows.

EXPERIMENTAL PROCEDURE

Eight Holstein and two Brown Swiss cows from the Cornell University Dairy Herd were used in this study. These cows were paired according to breed, age, and previous production. The cows were placed on the experiment on the 2nd wk. after calving. The 1st wk. after calving served as a preliminary period.

A double-reversal experiment was used, consisting of six experimental periods. Each period consisted of two weeks, with no transitional period between the experimental periods. Two types of grain were fed during the experimental periods. The first type was an 18% protein pelleted grain mix containing 5% sodium propionate. Poundages of composition were as follows: linseed oil meal and soybean oil meal, 200 each; gluten feed, distillers grains, and sodium propionate, 100 each; wheat bran, 300; ground oats, 500; hominy, 450; salt, 20; calcite, 10; dicalcium phosphate, 15, and trace minerals, 5. The other type of grain was the same mixture, except that the propionate was replaced by hominy feed. This served as the control feed.

One member of each pair was started on the control feed and the other member on the propionate feed. At the end of 2 wk. the type of grain was changed, and this was repeated every 2 wk. until a cow had been on three experimental periods of propionate feed and three periods of control feed.

Each cow received 10 lb. of propionate or control feed daily. During the propionate periods each cow received ½ lb. of sodium propionate from these 10 lb. of grain. The amount of grain required was determined from Appendix Table VIIIa in Morrison *(8)*. The difference between the 10 lb. of the basal grain mix fed and the total amount of grain required daily was made up of the regular herd mix containing 16% protein. The composition of this in pounds was as follows: ground oats, 460; ground corn or hominy, 400; wheat bran, 300; corn gluten feed, 500; soybean oil meal, 200; cane molasses, 100; iodized salt and steamed bone meal, 20 each; also, there was 2 g. of cobalt salt.

The amount of grain fed was changed at 4-wk. intervals, to insure that the same amount of grain was fed during one period of propionate feeding and one control period. An upper limit of 20 lb. of grain daily was set for all cows, to insure complete grain consumption. Each cow received 36 lb. of corn silage daily, and a mixed hay containing approximately 30% legume was fed ad libitum. Daily weigh-backs were taken on all feed, to insure accurate records of feed consumption.

Daily milk weights were taken and weekly composite milk samples were analyzed for butterfat content by the Babcock method. Blood samples were taken from the jugular vein at weekly intervals.

Potassium oxalate was used to prevent coagulation of the blood. The proteins were precipitated by the method described by Folin and Wu *(5)* and the blood sugar was determined colorimetrically by the method of Benedict

(2). Blood ketones were distilled from the filtrate by the method of Behre and Benedict *(1)* and the ketone bodies were determined as acetone by the method of Block and Bolling *(3)*.

Three-day weights were taken on all cows before they started on the experiment and before they were switched from one feed to another.

In a supplemental experiment, two dairy steers with rumen fistulas were used to determine the rate of disappearance of the propionic acid from the rumen. The steers were fed 5 lb. of control feed and 5 lb. of hay twice daily for 1 wk. At the end of a week, the fatty acids in the rumen were determined. A rumen sample was taken in the morning before the animal was fed and at hourly intervals after feeding for 10 hr. The 2nd wk., the steers received the same amount of propionate feed and hay, and at the end of the 2nd wk. the fatty acids in the rumen were determined by the same method.

The rumen samples were processed, distilled, and evaporated by the method described by Card *(4)*. The volatile fatty acids were separated according to the procedure of Marvel and Rands *(6)*, as modified by Card *(4)*.

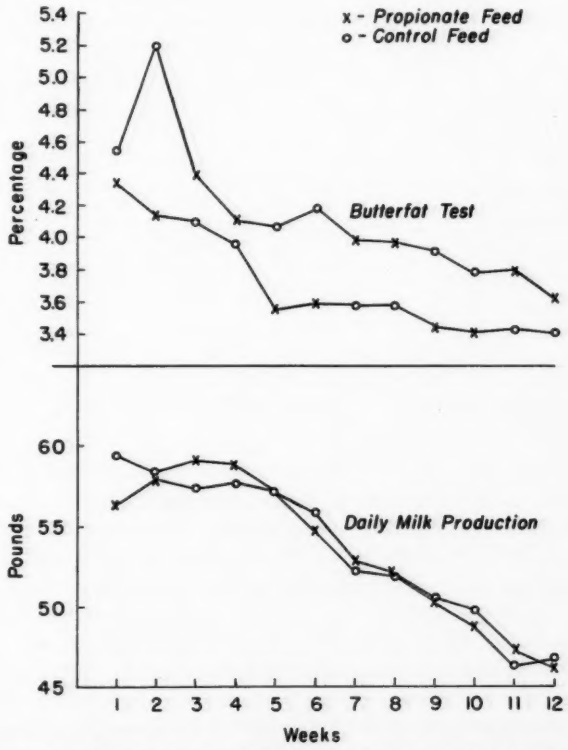

FIG. 1. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on milk production and butterfat test.

RESULTS AND DISCUSSION

The average daily milk production by weeks is given (Figure 1). The daily feeding of ½ lb. of sodium propionate did not affect the milk production. The cows produced an average of 53.6 lb. of milk daily on the control feed and 53.5 lb. on the propionate feed. These results indicate that the stimulation in milk production observed following the feeding of propionate to ketosis cows was a result of a return to normal rather than a stimulation above normal.

A slight reduction in the butterfat tests occurred on the propionate feed. The weekly butterfat tests are also given (Figure 1). The feeding of propionate caused an average reduction of 0.11% in the butterfat tests during the entire experiment (Table 1). Analysis by the "t" test showed that this was not statistically significant. The high peak in the butterfat test during the 2nd wk. of control feeding was the result of two cows having exceptionally high butterfat tests. The butterfat tests for the first 6 wk. were more variable than for the last 6 wk.

TABLE 1

Summary of the effects of feeding one-half lb. of sodium propionate daily to dairy cows during alternate 2-wk. periods after calving

Measurement	Average values[a] for three 2-week periods on: Control feed	Propionate feed
Milk production *(lb/cow/day)*	53.6	53.5
Milk fat test *(%)*	3.98	3.87
Total fat *(lb/cow/period)*	30.1	29.1
Hay intake *(lb/cow/day)*	14.6	14.6
Silage intake *(lb/cow/day)*	35.0	35.3
Grain intake *(lb/cow/day)*	17.3	17.3
Blood glucose *(mg. %)*	50.0	50.7
Blood ketones *(mg. %)*	5.8	4.8
Body weight changes *(lb/cow/period)*	+0.1	−0.8

[a] Differences due to propionate were not statistically significant in the case of any of the measurements.

The effect of propionate feeding on blood sugar and blood ketone levels is shown (Figure 2). The general conclusion that can be drawn is that when the blood glucose and ketone values were normal, propionate feeding caused no appreciable change. However, when these values varied from the normal in the direction of ketosis, propionate feeding tended to cause them to return to normal. During the first 2-wk. period, the cows started on the control feed showed above-normal blood ketone levels and slightly low blood sugar values. The milk fat test was also unusually high (Figure 1). The feeding of propionate during the next 2-wk. period appeared to bring all of these measurements back toward normal. During the rest of the experiment, the blood sugar and ketone values were within the normal ranges and propionate feeding caused no appreciable changes.

The average values of the various measurements taken during the course of the experiment are given (Table 1). Milk production and feed consumption were practically identical on the control and propionate feeds. Milk fat percentage and total fat production were reduced by the propionate feeding, but the differences were not statistically significant. Average blood ketone values were slightly lower and there was a very slight weight loss while the cows were being fed the propionate. Again, the small differences were not statistically significant. Most of the cows lost weight during the first 6 wk. of the experiment and gained during the last 6 wk.

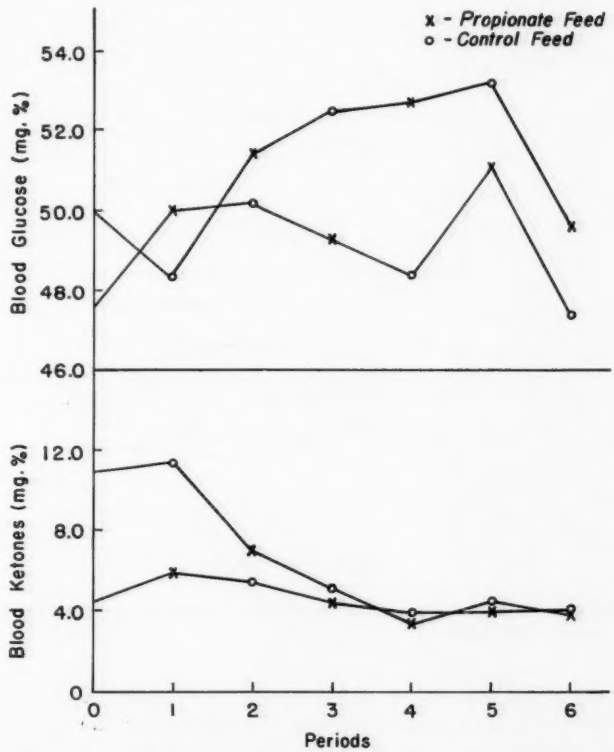

FIG. 2. Effect of feeding ½ lb. of sodium propionate daily during alternate 2-wk. periods on blood sugar and blood ketones.

The results of the supplemental experiment, designed to determine what happened to the fed propionate and how it affected the normal rumen fermentation, appear in Figure 3. The control feed showed the normal curve of fatty acid content in the rumen following feeding, with peak concentration 3 hr. after feeding. When the propionate feed was fed, the level of propionic

acid logically was much higher when the first sample was taken 1 hr. after feeding. It is of interest to note that the levels of acetic and butyric acid were also higher. It is doubtful whether this could be interpreted to mean that the propionate stimulated the production of the other acids, but certainly

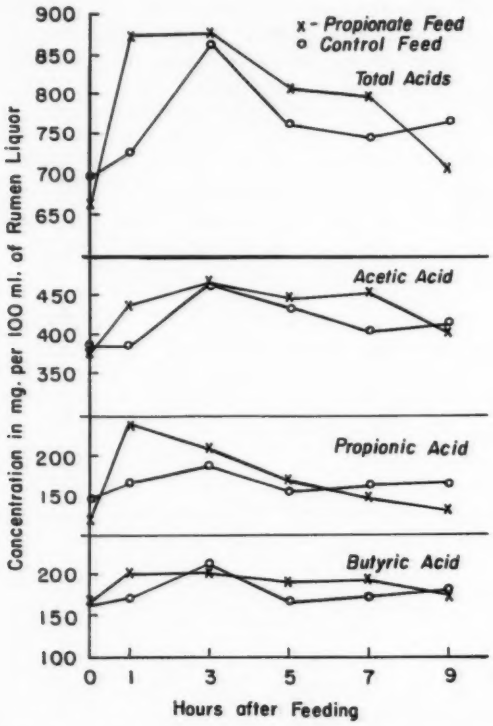

FIG. 3. Effect of feeding 5 lb. of grain containing 5% sodium propionate on volatile fatty acid concentration in the rumen.

it is clear that it did not depress their normal production. After the 3-hr. peak concentration was reached in each case, the curves for the control and propionate feeds followed each other closely. It was calculated that all the administered propionate had disappeared in 7 hr., with most of it disappearing within 3 hr.

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GROUND CORN COBS AS A SOURCE OF ROUGHAGE FOR LACTATING DAIRY COWS[1]

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SUMMARY

Two studies were conducted with milking cows to compare ground corn cobs with alfalfa hay for roughage, and a high protein (20.8 to 22.6%) grain mix was fed to supply Morrison's *(9)* minimum TDN standards, assuming that the corn cobs had the same TDN value as the alfalfa hay. In Trial I, corn cobs were substituted for hay at the rate of 8.0 and 16.0 lb. per day without affecting milk production, fat test, or body weight. An alfalfa-brome grass hay, which had a TDN value of 47.8%, was used for comparison.

In Trial II, corn cobs replaced alfalfa hay up to 60% of the total roughage intake without affecting milk production, and up to 80% of the total roughage without seriously affecting milk production. The TDN content of the alfalfa hay was 52.0 to 53.0%. When the cows were fed 0.4 lb. of hay and 2.1 lb. of corn cobs per 100 lb. of body weight, consumption of total roughage decreased 1.6 lb. per cow daily. Corn cobs appear to be equal to medium-quality alfalfa hay up to the limit of the cow's ability to consume them. Digestion trials were conducted in both studies, but no significant trends were noted.

The use of ground corn cobs as a roughage for beef cattle has been well established *(1, 2, 3)*. Fries *et al. (4)* of the Kentucky station and Harshbarger *(6)* of the Illinois station have reported data showing that corn cobs can be used as a satisfactory roughage for growing heifers. Hill *et al. (7)* of the Purdue station reported data which indicated that corn cobs were not a satisfactory source of roughage for milking cows. Cows fed corn cobs as the sole source of roughage declined 53.4% in milk production over a 12-wk. trial, as compared to a 41.5% decline for cows fed alfalfa hay and silage as the roughages. Both groups were fed "Purdue Supplement A" as the grain mixture. Cows fed corn cobs, however, gained 93.3 lb. in weight as compared to 16.0 lb. for the alfalfa hay and silage cows. The total dry matter consumption per 100 lb. of body weight was 2.36 lb. per day for the cows fed corn cobs, and 2.76 lb. for the alfalfa hay and silage fed cows. Graf and Engel *(5)* of the Virginia station reported the feeding of a limited amount of corn cobs, 5.5 lb. per day, in place of an equivalent amount of alfalfa-grass hay. The same amount of corn silage and grain was fed to each group. Cows fed no corn cobs produced 22.0 lb. of milk more per group than the cows receiving corn cobs, over a 30-day period. Cows fed corn cobs gained slightly more in weight than the cows fed hay. Digestion trial data favored slightly the cows fed corn cobs.

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The purpose of this experiment was to further evaluate the use of ground corn cobs as a source of roughage for milking cows, and particularly to determine the extent to which corn cobs could be used as a substitute for hay.

EXPERIMENTAL PROCEDURE

Two studies have been made concerning the use of ground corn cobs as the source of roughage for milking cows.

Trial I. Fifteen lactating Holstein cows were used in five 3 × 3 Latin-square designs. Each period was 27 days in length. Group 1 received 25.0 lb. per day of a second-cutting alfalfa-brome grass hay; Group 2 received 17.0 lb. of hay and 8.0 lb. of corn cobs, and Group 3 received 9.0 lb. of hay and 16 lb. of corn cobs. The total digestible nutrient (TDN) content of the hay was 47.8%. All cows were fed a simple grain mixture composed of 1,260 lb. of yellow corn, 700 lb. of soybean oil meal, 20 lb. of trace salt containing idoine and cobalt, and 20 lb. of dicalcium phosphate. Grain was fed at a rate to supply Morrison's *(9)* minimum TDN standards. Adjustments in grain feeding were made at the end of each period based upon the average decline in milk production of all cows *(8)*. Milk weights were recorded daily and samples for butterfat percentage were taken from each milking and composited on a three-day basis. Digestion trials were conducted with seven cows receiving comparable amounts of feed during each of the three periods, using chromic oxide as the indicator. Grab samples of feces were collected twice daily, at 6 A.M. and 4 P.M. for seven days after the cows had been on the rations for a minimum of ten days. All cows were weighed every third day throughout the study.

Trial II. Sixteen lactating cows (12 Holsteins and four Jerseys) were divided into four comparable groups and used in 4 × 4 Latin-square designs. Each period was 28 days in length, to compare four levels of hay and corn cobs as roughages for milking cows. All cows were fed roughage at a rate of 2.5 lb. per 100 lb. of initial body weight. The rations were as follows: Group 1—2.5 lb. of hay per 100 lb. of body weight; Group 2—1.8 lb. of hay and 0.7 lb. of corn cobs per 100 lb. of body weight; Group 3—1.1 lb. of hay and 1.4 lb. of corn cobs per 100 lb. of body weight, and Group 4—0.4 lb. of hay and 2.1 lb. of corn cobs per 100 lb. of body weight. Two alfalfa hays were used in the study. During Periods 1 and 2, a prebloom alfalfa hay with TDN content of 53.0% was used, and during Periods 3 and 4, an excellent quality third-cutting alfalfa hay with a TDN content of 52.0%. A grain mixture composed of 1,210 lb. of yellow corn, 750 lb. of soybean oil meal, 20 lb. of a trace salt containing iodine and cobalt, and 20 lb. of dicalcium phosphate was fed as outlined in Trial I. Body weights were recorded for three consecutive days at the beginning and at the end of each period. Daily milk weights were recorded and samples for butterfat percentage were taken from four consecutive milkings each week. Digestion trials were conducted on all cows during the last seven days of each period, according to the procedure used in Trial I. The corn cobs in both studies were ground through either a 0.625 or a 0.5 screen.

RESULTS AND DISCUSSION

Composition of the experimental feeds for both Trials I and II is shown (Table 1). In Trial I, the hay was 0.5 bloom alfalfa-brome and was slightly rain-damaged, which accounts for its slightly low TDN value. The grain mixtures

TABLE 1
Composition of experimental feeds

Feed	Dry matter	Crude protein	Crude fiber	Ether extract	N-free extract	Ash	TDN[a]
	(%)						
				Trial I			
Alfalfa-brome hay	88.1	13.4	34.7	1.2	33.0	5.8	47.8
Corn cobs	81.4	3.3	25.6	0.8	50.0	1.2	.....
Grain mix	87.1	20.8	3.8	3.1	54.8	4.6	.....
				Trial II			
Alfalfa hay[b]	89.2	17.6	28.1	1.9	34.6	7.0	53.0
Alfalfa hay[c]	84.4	15.3	28.1	1.4	33.8	5.8	52.0
Corn cobs	84.1	2.0	30.3	0.2	50.2	1.4	.....
Grain mix	87.9	22.6	3.0	2.7	54.5	5.1	.....

[a] Actual TDN values.
[b] Used in first part of Trial II.
[c] Used in last part of Trial II.

in both Trials I and II were calculated to supply enough protein to meet the cows' requirements when receiving the least amount of hay and the largest amount of corn cobs; thus, when the cows were fed a high proportion of hay, an excessive amount of protein was fed.

The consumption of feed in both trials is presented (Table 2). None of the differences in roughage or grain consumption among groups was statistically

TABLE 2
Summary of feed consumption
(Av. intake/cow/day)

Ration	Hay[a]	Corn cobs[a]	Grain[a]
		Trial I	
A	24.9	.....	8.7
B	17.0	7.8	8.7
C	9.0	15.3	8.5
		Trial II	
1	27.8	.....	6.6
2	20.1	7.7	6.6
3	12.2	14.9	6.7
4	4.5	21.7	6.8

[a] No significant differences.

significant; however, as the level of corn-cob feeding increased, there was a tendency for the total consumption of roughage to decrease. Cows on Ration C (Trial I) consumed a total of 24.3 lb. of roughage per day, compared to 24.9 lb.

for Ration A and 24.8 lb. for Ration B. In Trial II, the cows on Ration 4 consumed only 26.2 lb. of total roughage per day, compared to 27.1 lb. for Ration 3, and 27.8 lb. for both Ration 1 and Ration 2. The decrease in corn-cob consumption was not the result of a few cows refusing to eat corn cobs but of a general lowering of corn-cob consumption by all cows. The grain mixtures used in these studies did not include molasses, which may have improved the consumption of corn cobs.

Effect of the various levels of corn-cob feeding on milk production and body weight changes is presented (Table 3). The feeding of corn cobs did not appear to have any appreciable effect on the average butterfat percentage in any trial.

TABLE 3

Effect of feeding corn cobs as a partial replacement of hay on the milk production and body weight changes of dairy cows

Ration	Av. test[a]	Av. 4% FCM[a]		Average body weight change[a]
		7 to 28 days	Last 7 days	
	(%)	*(lb/day)*		*(lb/cow)*
		Trial I		
A	3.5	33.1	32.6	—3.7
B	3.5	33.8	34.3	9.0
C	3.7	33.6	33.7	4.7
		Trial II		
1	4.0	29.2	28.4	9.9
2	3.9	28.3	27.8	3.7
3	4.0	28.6	27.7	22.2
4	4.1	27.2	26.2	16.4

[a] No significant differences.

This was true even with the cows fed Ration 4 (Trial II), when only 4.5 lb. of hay was consumed daily. In Trial I, the feeding of either 8 or 15 lb. of corn cobs per day did not significantly affect milk production. Similar observations were made in Trial II; however, the milk production of cows fed Ration 4 was lower than the other three groups, even though these differences were not statistically significant. The feeding of corn cobs did not significantly affect body weight changes, although the cows fed high amounts of corn cobs tended to make the greatest body weight gains. This observation is in agreement with both the Purdue *(7)* and Virginia *(5)* workers.

Data (Table 4) show the effect of the various corn-cob rations on the over-all digestibility of the rations. In Trial I, the digestibility of protein was significantly higher in Ration A than in either Ration B or Ration C. Possibly, this could be due to the higher level of protein intake in this ration. Crude fiber digestion was higher with Ration C than with either A or B, but these differences were not significant. Dry matter digestibility did not appear to be influenced significantly.

In Trial II, the coefficients of digestibility were similar to those obtained in Trial I. No significant differences existed in crude fiber digestibility among

rations, but the dry matter coefficients for Rations 1 and 2 were higher than for Rations 3 and 4. The coefficients obtained for Ration 3 were higher than those for Ration 4. Similar trends were noted for crude protein digestion which were probably due to a lower intake of crude protein in Rations 3 and 4. No significant difference existed among rations in respect to crude fiber digestibility coefficients.

TABLE 4

Effect of various levels of corn-cob feeding on the apparent digestibility of the over-all ration

Ration	Dry matter	Crude protein	Crude fiber	Ether extract	N-free extract
			(%)		
			Trial I		
A	56.6	65.0	45.4	35.1	65.0
B	52.2	59.9	44.6	34.8	58.4
C	56.2	58.7	51.8	48.2	59.7
L.S.D. —5%	NS	4.39	NS	.....	.....
			Trial II		
1	61.3	67.5	41.7	55.3	67.1
2	60.0	67.2	43.4	56.2	62.8
3	57.3	59.7	37.8	57.2	60.5
4	54.9	44.5	37.8	56.3	58.4
L.S.D. —1%	.....	3.70	NS	.....	.....
L.S.D. —5%	2.12	.....	NS	.....	.....

The results of these two studies concerning the nutritive value of ground corn cobs as a source of roughage for milking cows indicate that when corn cobs are properly supplemented, they contain more feeding value than is commonly recognized. Results of Trial I showed that corn cobs were equal to medium-quality hay when fed to the limit of substitution for hay used in this study. Trial II was conducted primarily to determine the extent to which corn cobs could substitute for hay as a source of roughage in the ration of milking cows, and also to compare corn cobs with a higher quality hay (TDN) than was used in Trial I.

Data obtained in Trial II show that corn cobs can be substituted up to about 60% of the total roughage intake without affecting milk production. It should be pointed out that a very high-quality hay (52.0-53.0% TDN) was used in Trial II and was substituted by corn cobs on an equal pound basis. This means that if a slightly poorer hay—which would probably be the quality of hay on most dairy farms—had been used as a comparison, corn cobs would have compared more favorably with hay. It appears that corn cobs are equal to average-quality hay on a per-pound basis up to the limit of the cows' ability to consume corn cobs. In Trial II, consumption of corn cobs was not equal to hay, when corn cobs composed 80% of the total roughage intake.

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GROWTH STUDIES WITH CALVES AND RABBITS FED TIMOTHY HAY GROWN ON HEAVILY FERTILIZED SOILS [1, 2]

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SUMMARY

This experiment was conducted to identify nutritional deficiencies in timothy hay grown on a heavily fertilized soil. When this hay was fed to calves with a concentrate mixture of degermed corn meal, nonfat dry milk solids or urea, vitamins A and D, cobalt, calcium, phosphorus, and common salt, the ration was deficient in iron, copper, and iodine, resulting in poor growth, poor appetite, anemia, and enlarged thyroids. Feeding iron and copper, either individually or in combination, did not correct these symptoms. Iodine supplementation corrected the thyroid condition but did not stimulate growth. When similar hay was fed to rabbits in a ration supplemented with wheat gluten, hydrogenated vegetable oil, dextrose, sodium chloride, and vitamins A and D, it also resulted in poor growth, anemia, and increased thyroid weight. Feeding of a ration containing 5% alfalfa prevented all these symptoms, but an equivalent amount of alfalfa ash or iodine prevented only the enlarged thyroid. The timothy hay apparently was deficient in iodine and some organic factor(s) which may be found in alfalfa hay.

An experiment which has been in progress at the New Hampshire Agricultural Experiment Station for a period of several years has shown that certain forage crops grown under conditions of heavy applications of NPK fertilizers to the soil may be low in iron, copper, cobalt, and magnesium. When such forages were fed to dairy calves, along with a low trace mineral concentrate mixture containing supplemental vitamins A and D, calcium, phosphorus, cobalt, and common salt, they resulted in poor growth, anemia, sagging of the spinal column behind the shoulders, and other symptoms. In mature animals, the feeding of this type of ration has resulted in rapid loss of flesh after calving, poor milk production and, in many instances, in the production of goitrous calves. Rabbits fed similar forages have shown poor growth, mild anemia, and hyperplastic thyroids.

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[2] This study was supported in part by a research grant from Central Laboratories, General Foods Corporation, Hoboken, N. J. The vitamin A acetate was contributed by the Chas. Pfizer Co., Inc., Terre Haute, Ind., and the crystalline urea by E. I. duPont de Nemours and Co., Wilmington, Delaware. This study was carried out as a subsidiary phase of a project entitled "The Effect of Trace Mineral Deficiencies on the Reproductive Performance of Dairy Cattle." This main project was contributory to the regional research project, NE-1, entitled "Causes and Prevention of Reproductive Failure in Dairy Cattle."

While the need for supplemental cobalt under such conditions in New Hampshire has been well established *(1)*, a need for iron and copper has not. It was the purpose of the experiments reported here to determine whether iron and copper were lacking in the ration of calves, and to determine the responsiveness of rabbits fed these deficient rations. In supplementary studies iodine, zinc, and alfalfa were tested for growth-stimulating properties.

EXPERIMENTAL PROCEDURE

Calf studies. The forage studied in this experiment was first-cutting timothy hay, harvested in 1954 from a Paxton loam. It was seeded in 1950 on land which was limed before seeding and fertilized each year with a 7–7–7 mixture, applied at the rate of 600 lb. per acre in April and 400 lb. per acre after the first crop was removed. The hay was harvested in the early-bloom stage, cured and baled in the field.

The experimental animals used were 16 Guernsey heifer calves purchased from well-fed herds. The calves were kept in individual wooden pens and were bedded with shavings and watered from aluminum pails. In the pre-experimental or depletion period, the calves were weaned from whole milk or milk containing concentrates, to a hay and grain concentrate regimen, in the following manner: Whole milk was fed for approximately 6 wk., followed by reconstituted skimmilk. This was replaced gradually with a concentrate mixture containing 33 lb. nonfat dry milk solids, 66 lb. degermed corn meal, 1 lb. NaCl, 500,000 units of vitamin A as vitamin A acetate, and 142,000 units of vitamin D as irradiated yeast. When the calves averaged 8 mo. of age, the concentrate mixture was changed to 100 lb. degermed corn meal, 3 lb. crystal urea, 1 lb. NaCl, 500,000 units of vitamin A, and 142,000 units of vitamin D. Both of these mixtures contained approximately 16% crude protein equivalent. Cobalt was fed to all animals throughout the experiment, at the rate of 12.5 mg. twice a week as a drench.

The experimental animals were divided into four replications containing four animals each. Within each replication, one animal was fed 156 mg. $CuSO_4 \cdot 5H_2O$ per 100 lb. body weight twice a week. Another received 397 mg. Fe from carbonyl iron powder per 100 lb. body weight twice a week. A third animal received the same amounts of both of these elements, and the fourth animal was kept as a control. These amounts of iron and copper were approximately those reported by Knoop *et al. (2)* as meeting the requirements of dairy calves. Mineral supplementation was started when the calves averaged 4 mo. of age. Gelatin capsules were used as the method of feeding. After 2 mo., the rates of iron and copper fed were increased to four times the original levels. This change was made because the anemic condition of the calves had not been corrected, and it was felt that relatively large amounts of both iron and copper should be fed to make sure that the intake of these elements was adequate. Hay and basal concentrate mixture were fed ad libitum to the calf with the lowest feed consumption in each replication. The other animals in each replication

were given the amounts of feed consumed by that animal. As the experiment progressed, it became impossible to keep hay and concentrate consumption in balance. Beginning when the animals averaged 12 mo. of age, the hay was ground and mixed with the concentrate in a 2 to 1 ratio.

By the time the experimental animals had reached 14 mo. of age, it had become obvious that no response to iron or copper could be expected. It was decided at this time to try other treatments superimposed upon the iron and copper supplementation, and to allow ad libitum feeding. At this time, the members of one replication were injected with radioactive iodine and then slaughtered, to measure thyroid functioning. Immediately after this, iodine was fed to the members of one of the remaining replications at the rate of 5.25 mg. KI twice a week as a drench. Zinc at the rate of 302 mg. $ZnSO_4 \cdot 7H_2O$ per 100 lb. body weight was added to the daily ration of this same group. A second replication was fed this amount of zinc without iodine, and the members of the third replication were kept as controls. After a period of 3 mo., 25% of the concentrate mixture of those animals getting both iron and copper and those getting neither was replaced with dehydrated alfalfa pellets purchased in Kansas, and the previous treatments were continued. This was done to see if the growth-promoting properties of alfalfa, as evidenced in the experiment carried out with rabbits, could be observed. These treatments were continued for a period of 2.5 mo., until all of the animals were slaughtered and their thyroids removed and weighed.

Rabbit studies. The timothy hay used in these studies was harvested in 1953 from the same fields as the hay used in the calf studies. It was ground in a hammer mill to pass a 3/8-inch screen. The composition of the basal diet in percentages was, ground timothy, 75; wheat gluten, 10; hydrogenated vegetable oil, 7.9; dextrose, 6; NaCl, 1, and A and D percomorph oil, 0.1. All supplements to this diet were made at the expense of the dextrose. A diet in which ground alfalfa hay replaced the timothy of the basal diet served as a control.

Dutch belted rabbits 4 wk. of age were selected from a group weaned at 3 wk. of age and that had had access to the basal diet only from the age of 2 wk. The rabbits were individually caged and fed ad libitum. Double-demineralized water was offered the animals throughout the experiment. A randomized block design with six replications was used with rabbits, which were randomized to the treatments from weight-sex groups.

In one experiment, treatments included the following supplements to the basal diet: alfalfa ash (0.4 g. per 100 g. diet), cation and anion fractions obtained from an equivalent amount of alfalfa, and potassium iodide (25 μg. per 100 g. diet). In another experiment, the basal diet was supplemented with 5% alfalfa (second cutting, 1954 crop from Ithaca, New York).

EXPERIMENTAL RESULTS WITH CALVES

Composition of feeds. The chemical composition of the feeds included in the basal rations fed the calves is shown (Table 1). These rations were low in

iron, copper, cobalt, iodine, and magnesium. Zinc was relatively low, but a requirement has not been established. Calcium and phosphorus intakes were low before the addition of dicalcium phosphate, and particularly low following the change to urea. Magnesium was not added to the ration because deficiency levels were not found in the blood of these animals or others fed similar rations.

TABLE 1
Composition of feeds

	Timothy hay	Degermed corn meal	Nonfat dry milk solids[a]
Protein *(%)*	4.24	8.06	33.5
Fat *(%)*	1.74	0.36	0.9
Fiber *(%)*	32.97	0.32	0.2
Ash *(%)*	3.49	0.26	7.6
H_2O *(%)*	8.32	10.98	6.1
Ca *(%)*	0.25	0.03	1.26
P *(%)*	0.13	0.04	1.03
Fe *(p.p.m.)*	36.7	4.5	52.0
Cu *(p.p.m.)*	2.6	1.3	11.5
Mn *(p.p.m.)*	56.7	1.5	2.2
Co *(p.p.m.)*	0.03	0.02	0.11
Zn *(p.p.m.)*	17.8	2.9	—
I *(p.p.b.)*	35.0	34.0	—
K *(%)*	1.39	0.12	1.67
Mg *(%)*	0.07	0.01	0.11

[a] Values taken and calculated from *Composition of Concentrate By-Products Feeding Stuffs*. National Academy of Sciences. National Research Council Publ. 449. June, 1956.

Feed consumption and growth. Average body weights and TDN consumption for the heifers in the various treatments are shown (Figures 1 and 2, respectively). It can be seen that the animals in all treatments grew at a moderate but subnormal rate until the nonfat dry milk solids were removed from the concentrate mixture and urea was added. This change resulted in a rather marked decrease in feed consumption and growth rate. When the animals were changed to the ground hay–concentrate mixture, nutrient intake was depressed still further and the animals lost weight. In general, the rate of growth was simply a reflection of nutrient intake expressed as calculated TDN. All evidence from this and related studies indicates that feed consumption was low because of the lack of an appetite-stimulating factor, not because of the feed being particularly unpalatable. It appears that this factor was most deficient in the ration containing urea.

During the entire period of the experiment, there was no indication that either copper or iron alone, or together, stimulated appetite or increased body weight gains even at levels of approximately four times the published requirements. This does not mean necessarily that iron and copper were not deficient but that a deficiency of some other factor may have prevented a response.

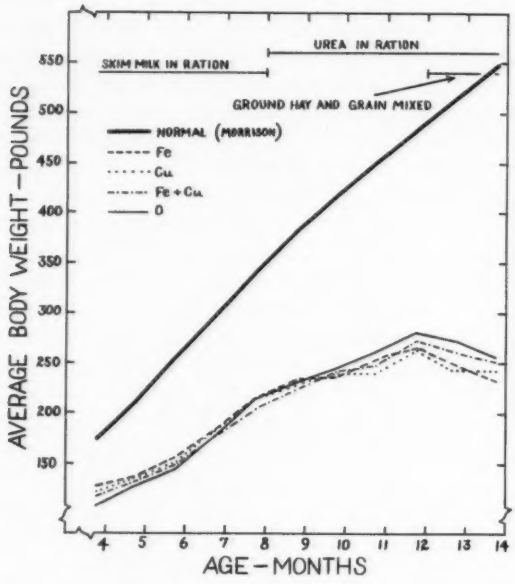

FIG. 1. Average body weights of calves fed various different mineral supplements.

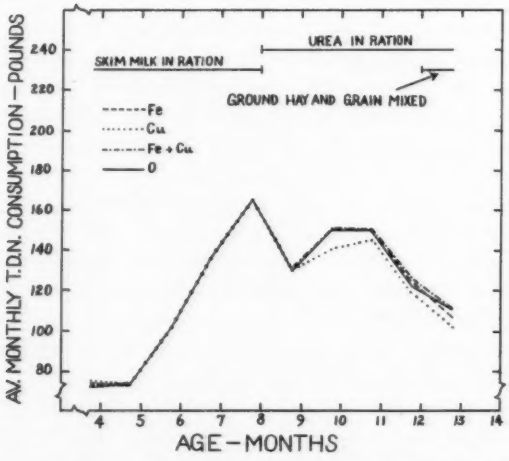

FIG. 2. Average total digestible nutrient consumption of calves fed different mineral supplements.

Inorganic phosphorus of blood. After the experiment got under way, it was observed that serum inorganic phosphorus dropped more rapidly in the control animals than in those getting either iron or copper, or both. These results are shown (Figure 3). Differences between the controls and those fed copper alone, or copper and iron, were significant at the 5% level. Differences between animals fed iron and copper, or iron alone, were significant at the 5% level.

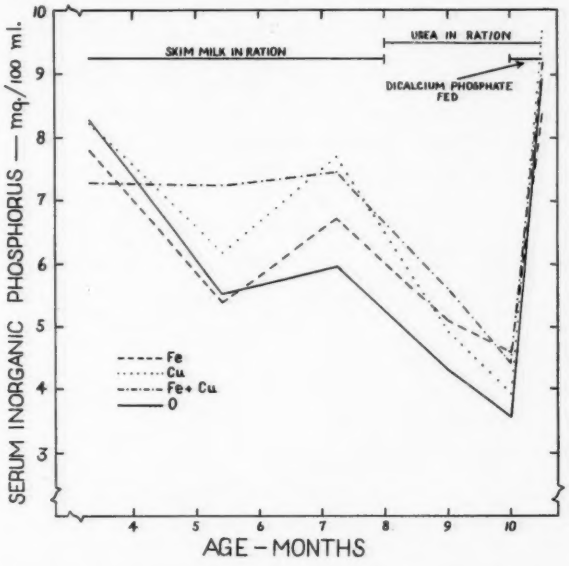

FIG. 3. Average serum inorganic phosphorus levels of calves fed different mineral supplements.

After the removal of nonfat dry milk solids from the ration, phosphorus deficiency developed so quickly that one animal was injured in the rear quarters and had to be removed from the experiment. After this happened, all animals were fed 40 g. of dicalcium phosphate per day, and differences due to iron or copper supplementation disappeared and serum inorganic phosphorus levels returned to normal. These results suggest that under conditions of low phosphorus intake, both iron and copper may influence the level of inorganic phosphorus in the blood, and the combination appears to exert a greater influence that either one alone. However, supplementation of the ration with phosphorus did not stimulate growth or feed consumption.

Hemoglobin levels. Hemoglobin levels were somewhat low throughout the experiment, with average values falling between 8 and 10 g. per 100 ml. of blood. There was no evidence that the feeding of iron, copper, or both, had any significant effect on hemoglobin level at any time during the experiment.

Supplementary studies. Results from the injection of radioactive iodine, which are being published elsewhere, showed that the uptake and conversion of this element were many times greater than normal. The feeding of zinc, or iodine and zinc, did not increase feed consumption or body weight above the controls. Subsequent studies with radioactive iodine showed that the feeding of iodine for a period of 2 mo. had corrected the abnormal thyroid functioning, although no other response could be observed. When the remaining animals were slaughtered at the end of the experiment, thyroid weights were found to be as given (Table 2). These data show a highly significant difference in thyroid weight between those animals which received iodine and those which did not. It appears that the increase in thyroid weight of the animals not fed iodine took place after the first animals were slaughtered. Copper supplementation appeared to increase thyroid size slightly (not significant at 5% level), but iron, zinc, and alfalfa had no observable effect on thyroid size.

TABLE 2

Thyroid weights of calves

Replication No.	Animal No.	Supplement: Beginning at 4 mo.	Supplement: Added at 14.5 mo.	Age when slaughtered	Thyroid weight
				(mo.)	*(g.)*
3	135	Cu		14	14
	136	Fe + Cu		14	13
	137	O		14	10
	138	Fe		14	6
	Av.				10.7
1	127[a]	Fe + Cu	I + Zn	20	13
	128	Fe	I + Zn	20	10
	129	Cu	I + Zn	20	15
	130[a]	O	I + Zn	20	17
	Av.				13.7
2	131[a]	Fe + Cu		20	52
	132[a]	O		20	36
	133	Cu		20	—
	134	Fe		20	46
	Av.				44.7
4	139[a]	O	Zn	20	19
	140	Fe	Zn	20	—
	141	Cu	Zn	20	44
	142[a]	Fe + Cu	Zn	20	35
	Av.				32.7

All Fe alone	20.7	All Cu (incl. Fe + Cu)	26.6
All Cu alone	24.3	All no Cu	20.6
All Fe + Cu	28.2	All alfalfa	28.7
Controls	20.5	No alfalfa	28.7

[a] Received Kansas alfalfa after 17½ mo. of age.

The feeding of the alfalfa pellets seemed to result in considerable appetite and growth response in some animals, but little or no growth in others. This inconsistent response pattern might be explained on the basis of varying pituitary changes, comparable to those found in animals maintained on a similar feeding program. Such changes involving the tissues which produce growth hormone might be corrected only slowly, if at all. It seems, therefore, that further studies will have to be carried out in order to understand this problem.

EXPERIMENTAL RESULTS WITH RABBITS

Poor growth, mild anemia, and hyperplastic thyroids also were evidenced in the rabbits fed the heavily fertilized timothy hay (shown in Table 3). The hyperplasia of the thyroid gland was reduced by the feeding of alfalfa ash or iodine supplements, but not by the cation or anion fractions of the ash. Iodine analysis indicated that 100 g. of the basal diet contained approximately 10 γ of iodine, that the alfalfa ash supplement contributed 2 γ, and that the iodine was lost in the preparation of the cation or anion fractions. Because iodine supplements alone reduced the size of the thyroid gland, it would appear that the iodine content of the basal diet furnished less than the rabbit's requirement or that the availability of the timothy iodine was low.

TABLE 3

Studies of the timothy hay with rabbits

Treatment	Weight gain	Hb per 100 ml. blood	Thyroid per 100 g. body weight
	(g.)	*(g.)*	*(mg.)*
	Experiment 1		
Basal	564	9.9	11.4
+ Alfalfa ash [a]	593	9.5	8.4
+ Cations [a]	510	10.2	13.3
+ Anions [a]	589	9.5	12.3
+ Iodine [b]	528	8.9	7.4
Control [c]	1,103	12.0	6.9
	Experiment 2		
Basal [a]	695	10.8	
Alfalfa [a]	965	12.8	

[a] At a level of 5% alfalfa, or its equivalent in ash (500° C. for 16 hr.). Added at the expense of dextrose in basal diet.

[b] 26 μg. per 100 g. diet.

[c] Alfalfa replacing timothy in basal diet.

The supplementation of the basal diet with 5% alfalfa improved body weight gain and hemoglobin formation in the rabbits; the ash of a comparable amount of alfalfa had no effect on these variables. This result suggests that alfalfa contains an organic factor or a volatile mineral other than iodine which is essential to the adequate nutrition of the rabbit. That alfalfa contains such a nutriment has been suggested by Thacker and Ellis *(3)*.

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ANAEROBIC PURINE DISSIMILATION BY WASHED SUSPENSIONS OF BOVINE RUMEN BACTERIA[1,2]

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SUMMARY

Xanthine, uric acid, and guanine hydrochloride were degraded completely, at a slow rate, by WCS of bovine rumen bacteria. The end-products formed were carbon dioxide, ammonia, and acetic acid, and the fermentation pattern was similar to that observed in clostridial-type organisms. Hypoxanthine was degraded incompletely, whereas adenine was neither decarboxylated nor deaminated under the conditions employed. Gases other than carbon dioxide (hydrogen and/or methane) never were encountered as end-products of purine degradation and neither were lactic, formic, propionic, and butyric acids. With the exception of hypoxanthine, WCS deaminated purines to a greater extent under alkaline than under acidic conditions. In experiments dealing with xanthine decarboxylation, less carbon dioxide was formed from this substrate than that observed for the corresponding endogenous value. This was not true of other purines. Kinetic treatment of the data suggested that a carbon dioxide utilizing or fixing mechanism was involved in xanthine dissimilation. Purine interconversions could not be demonstrated by paper chromatography. Differential spectrophotometric methods showed that xanthine and uric acid were degraded completely, hypoxanthine was partially degraded, and adenine was not dissimilated.

Purines are known constituents of plants and they exist in the free state or bound as nucleosides or nucleotides. Ferguson and Terry *(11)* reported the presence of purines in grassland herbage, i.e., adenine, guanine, xanthine, and hypoxanthine. The nonprotein nitrogen content of grassland herbage represents from 15 to 20% of the total nitrogen present, and from 50 to 60% of the nonprotein nitrogen fraction is ammonia, nitrate, acid amides, and amino acids. The remainder consists of purines, possible pyrimidines, either free or bound *(11)*. Guanine and adenine, well-known constituents of nucleic acids, have been found in the free state in alfalfa leaves *(28)*. Hypoxanthine, xanthine, and uric acid occur primarily in the free state, and uric acid has been observed in leaves and seeds in concentrations of 250 mg. per kilogram *(12)*. Therefore, free or bound purines are, in fact, ingested in feeds given to ruminants. Experiments were undertaken to determine to what extent catabolic activity was exhibited by washed cell suspensions (WCS) of bovine rumen bacteria on this group of compounds.

EXPERIMENTAL PROCEDURE

Rumen samples were obtained from a 4-year-old fistulated Holstein cow approximately four hours after morning feeding. The animal's diet consisted of U. S. No. 2 leafy alfalfa hay, ad libitum, supplemented daily with 16% protein concentrate. Methodology employed in processing and standardizing the WCS

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[1] Hereafter, WCS designates washed cell suspensions of bovine rumen bacteria.

[2] Scientific Article No. A632, Contribution No. 2814 of the Maryland Agricultural Experiment Station.

has been described previously by Doetsch *et al. (10)*. Total nitrogen determinations on WCS indicate that 1.0 ml. of the suspension was equivalent to 1.3 $\pm$0.2 mg. nitrogen. These cells were used for both manometric studies and long-term dissimilation reactions, described below.

Buffer solutions containing 0.067 *M* Na_2HPO_4 and KH_2PO_4 were combined in appropriate amounts to give the desired pH *(24)* immediately before use, then boiled and rapidly cooled and sufficient $Na_2S \cdot 9H_2O$ added to give a final concentration of 0.02% (w/v). Stock purine solutions were prepared by dissolving 5 m*M* in 10 ml. *N* NaOH in a 100-ml. volumetric flask, made up to volume with distilled water. These stock solutions of 0.05 *M* purines were diluted further with distilled water to the concentration levels desired. Immediately before use they were neutralized with HCl, the pH being adjusted to the desired value. All solutions were stored at 4° C. and the pH was checked electrometrically.

All experiments were performed under anaerobic conditions, atmospheric oxygen was displaced by thoroughly flushing the system with N_2, He, or a mixture of 1% (v/v) CO_2 in N_2. All gases were freed from oxygen by passage over reduced copper filings at 600° C.

Long-term dissimilation tests were modified as follows: *(a)* 50-ml. Erlenmeyer flasks were used with 60 μM purine substrate in 9 ml. of 0.067 *M* phosphate buffer at the desired pH [containing 0.02% (w/v) $NaS \cdot 9H_2O$], to which were added WCS and *(b)* 50-ml. Erlenmeyer flasks with 200 μM substrate in 12 ml. phosphate buffer, to which 8 ml. of WCS were added. Flasks were incubated at 39° C. in a water bath for periods ranging from 12 to 72 hr. Endogenous controls always were included and after incubation suitable inhibitors (depending on the type of analysis to be performed) were added to aliquots set aside for biochemical assay.

Manometric techniques (short-term dissimilation studies) also were employed, and carbon dioxide evolution and gases other than carbon dioxide evolved were determined using the direct method *(24)* and a modified Dixon-Keilin technique. In all instances, the fluid volume was 3.0 ml. and 0.5 ml. of purine (varying concentrations), and 0.2 ml. of 10 *N* H_2SO_4 containing 5% (w/v) perchloric acid were placed in the side-arms. Carbon dioxide was absorbed by 10% (w/v) KOH in the center well. Acid production also was demonstrated manometrically using bicarbonate-phosphate buffer under an atmosphere of 1% (v/v) CO_2 in N_2.

Dixon-Keilin flasks with two side-arms were employed with conventional Warburg constant-volume respirometers. Substrate (varying concentrations) and the acid-inhibitor were placed in the side-arms and KOH was placed in the stopcock opening which was exposed to the atmosphere of the reacting system. At the start, acid-inhibitor was tipped into one of the duplicate flasks giving initial "bound" carbon dioxide. After release of all bound carbon dioxide, the stopcock was turned, exposing the alkali to the atmosphere within the flask, thereby absorbing all carbon dioxide in the gaseous phase. This caused a drop in the manometer reading. The reaction was continued in the other

flask (duplicate) in the presence of carbon dioxide, causing a manometric change. At the end of a measured interval, acid-inhibitor was tipped into the second flask, which was similarly exposed to alkali. The total volume of carbon dioxide in each flask was estimated quantitatively. By use of this method, the following could be determined: initial and final bound carbon dioxide, initial and final total carbon dioxide, and final gases other than carbon dioxide (assuming the initial flask to have a value of zero). Reactions could be carried out in the presence of carbon dioxide within definite limits.

Lactic acid was determined colorimetrically *(2)*, and volatile fatty acids were determined by partition chromatography *(17)*. Known concentrations of volatile acids were used as markers with each series of fatty acid determinations, to verify identifications and quantitative estimations. Ammonia was estimated quantitatively by microdiffusion analysis *(9)*, as modified by Tonhazy *(23)*, as well as by direct titration with 0.02 *N* HCl. In the latter case, ammonia was evolved from an alkaline (pH 10) deproteinized (trichloroacetic acid, final concentration 10% w/v) aliquot by steam distillation (Pregl apparatus) and absorbed in 4% (w/v) boric acid solution containing a mixed sensitive indicator *(22)*. Ammonia standards were treated in the same manner, and in determinations on purine dissimilation, corrections for the endogenous were made by substraction. Total nitrogen was determined using a micro-Kjeldahl procedure *(18, 24)*. Protein nitrogen was determined indirectly as the difference between total nitrogen and nonprotein nitrogen. Deproteinization was carried out using trichloracetic acid [10% (w/v), final concentration] or perchloric acid [5% (w/v), final concentration].

Residual purine determinations were measured by following changes in ultraviolet absorption curves of deproteinized incubation mixtures at 260 and 280 mμ *(6, 25)*. Paper chromatography studies were carried out on purine interconversions, using one-dimensional chromatograms (Whatman No. 1 paper, sealed glass cylinder). These studies were performed at room temperature for 24 hr., using 77% (v/v) n-butanol, 10% (v/v) formic acid in distilled water *(19)*. Purine spots were identified by inspection under a "Mineralight" lamp possessing an ultraviolet filter emitting at approximately 250 mμ.

RESULTS

Deamination of xanthine, uric acid, and guanine hydrochloride occurred at pH 6.9 (as illustrated in Table 1). These purines were deaminated under neutral and alkaline conditions, and insignificant ammonia production was observed with hypoxanthine and adenine (under the same conditions). Excepting hypoxanthine, less ammonia was liberated from purines under acid conditions.

Studies on fatty acid production from xanthine are presented (Table 2). Acetic acid was the major volatile fatty acid end-product encountered. If assumed that when one mole of xanthine is degraded, one mole of acetic acid is formed, the results in Table 2 indicate that 90% of the xanthine was dissimilated. Volatile fatty acid production from other purines is summarized

TABLE 1

Anaerobic deamination of purines by rumen WCS[a]

Substrate	Initial ammonia	Final ammonia	Final minus initial	Corrected for endogenous	Theoretical ammonia	Theoretical ammonia
(0.01 M)	*(μM)*	*(μM)*	*(μM)*	*(μM)*	*(μM)*	*(%)*
Hypoxanthine	2.6[b]	24.2[c]	21.6	16.2	40	40
Xanthine	3.6	49.4	45.8	40.4	40	101
Uric Acid	3.6	46.2	42.6	37.2	40	93
Guanine HCl	2.4	51.4	49.0	43.6	50	87
Adenine	2.4	14.1	11.7	6.3	50	13
Endogenous	2.4	7.8	5.4	....	....	....

[a] 200 μM substrate, total volume 20 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM NH_3/ml initially present.
[c] Values represent μM NH_3/ml after 48 hr. incubation.

(Table 3). The greatest difference was noted with xanthine (425 μM of acetic acid was produced). This represents 103% dissimilation, assuming the formation of one mole of acetate from one mole of xanthine. Small increases in propionic and butyric acids were observed in this instance with xanthine. Negligible amounts of fatty acids were formed from hypoxanthine and adenine; whereas, small but definite fatty acid production was noted from uric acid and guanine hydrochloride. Formic acid never was observed in experiments on purine degradation.

Lactic acid never was observed as an end-product in purine degradation studies, regardless of the time of assay (from 3 to 72 hr.). There was no increase in protein nitrogen during purine dissimilation by WCS during long-term studies. If growth were a complicating factor, results would be difficult, if not impossible, to evaluate.

Paper chromatography studies yielded essentially negative results on purine interconversions. Rf values compared favorably with those reported by Markham and Smith *(19)*. The conversion of hypoxanthine, uric acid, and guanine hydrochloride to xanthine was not observed using this method.

Residual purine concentration was estimated quantitatively by differential spectrophotometry (as shown in Table 4). The per cent theoretical purine given in this table compares favorably with the per cent theoretical ammonia (shown in Table 1). A slight discrepancy existed with xanthine, because dif-

TABLE 2

Volatile fatty acid formation from xanthine by WCS[a]

Volatile acids	0.005 *M* xanthine	Endogenous	Difference
	(μM)	*(μM)*	*(μM)*
Valeric and higher	10.1[b]	12.7	....
Butyric	48.3	46.5	1.8
Propionic	67.0	64.3	2.7
Acetic	245.0	191.0	54.0
Formic	0.0	0.0	0.0

[a] 60 μM xanthine, total volume 12 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM volatile fatty acids per 12 ml. fermentation liquor.

TABLE 3

Volatile fatty acid formation from purines by WCS[a]

Substrate	Valeric and higher	Butyric	Propionic	Acetic
(0.01 M)	*(μM)*	*(μM)*	*(μM)*	*(μM)*
Hypoxanthine	Less than 6.0[b]	21.8	30.6	225
Xanthine	Less than 6.0	28.1	112.4	425
Uric Acid	Less than 6.0	25.0	96.8	263
Guanine HCl	Less than 6.0	18.8	100.0	258
Adenine	Less than 6.0	15.6	93.7	212
Endogenous	Less than 6.0	18.8	93.7	219

[a] 200 μM purines, total volume 20 ml., incubated 48 hr. at 39° C. under 100% N_2.
[b] Values represent μM volatile fatty acid per 20 ml. fermentation liquor.

ferential spectrophotometry gave a value of 92%; whereas, that obtained from ammonia and acetic acid formation was 101 and 103%, respectively.

Carbon dioxide production from purines was estimated manometrically and is shown (Figure 1 and Table 5). These short-term experiments indicated carbon dioxide evolution from uric acid, guanine hydrochloride, and hypoxanthine. Adenine was not decarboxylated by WCS. A definite decrease in carbon dioxide evolution consistently was observed with xanthine. Less carbon dioxide was formed from WCS plus xanthine, than from WCS alone. Because xanthine was degraded readily in long-term dissimilation studies as determined by ammonia production, volatile fatty acid formation, and disappearance of substrate (differential spectrophotometry), this phenomenon of decreased carbon dioxide evolution suggests a utilizing or fixing mechanism. Gases other than carbon dioxide (hydrogen and/or methane) never were formed in amounts substantially greater than the endogenous, in manometric studies.

Carbon dioxide and ammonia were estimated quantitatively, using varying concentrations of xanthine (as illustrated in Figure 2). Results of this experiment definitely suggest the involvement of a carbon dioxide fixing or utilizing mechanism. As the xanthine concentration increased, there was a marked decrease in the apparent decarboxylation rate and an increase in the deamination

TABLE 4

Residual purine determination from dissimilation studies with WCS[a]

Substrate	Incubation time	Optical density reading at: 250 mμ	260 mμ	280 mμ	Difference (260–250) or 260–280)	Residual purine	Theoretical purine
	(hr.)					*(μM/ml)*	*(%)*
Hypoxanthine	0	.....	0.648[b,c]	0.024	0.424	10.0	....
	48	.....	0.392	0.014	0.378	6.1	40
Xanthine	0	0.444	0.657	.....	0.213	10.0	....
	48	0.026	0.043	.....	0.017	0.8	92
Uric Acid	0	.....	0.040	0.135	0.095	10.0	....
	48	.....	0.067	0.058	0.009	0.9	90
Adenine	0	.....	1.208	0.459	0.749	10.0	....
	48	.....	0.927	0.280	0.647	8.6	14

[a] 200 μM substrate, total volume 20 ml., incubated at 39° C. under 100% N_2.
[b] Optical density of deproteinized aliquots diluted 1/100 with 0.1 *N* H_2SO_4.
[c] Optical density substracted for endogenous.

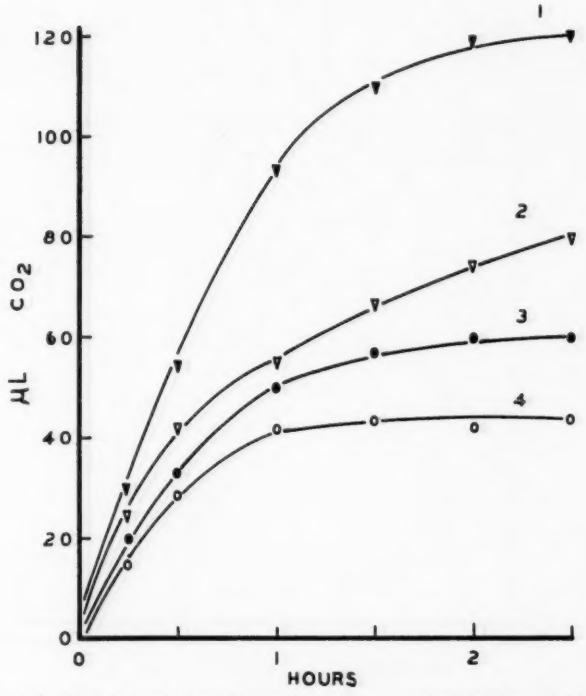

FIG. 1. Carbon dioxide evolution (determined manometrically) from 0.004 *M* purines: incubation 2.5 hr. at 39° C. under 100% N_2: 1, uric acid; 2, hypoxanthine; 3, endogenous; 4, xanthine.

rate. In addition, acid production from xanthine (Figure 3) was substantially greater than the corresponding endogenous value. To determine whether carbon dioxide influenced xanthine dissimilation, a modified Dixon-Keilin technique was employed. As observed from results (Figure 4), diminished carbon

TABLE 5

Manometric decarboxylation of purines by WCS [a]

Substrate	Initial carbon dioxide	Final carbon dioxide	Final minus initial	Corrected for endogenous
(0.0033 M)	*(μl.)*	*(μl.)*	*(μl.)*	*(μl.)*
Hypoxanthine	75[b]	150[c]	75	36
Xanthine	70	90	20	−19
Uric Acid	120	220	100	61
Guanine HCl	68	159	91	52
Adenine	65	107	42	3
Endogenous	61	100	39	....

[a] 10 μM substrate, total volume 3.0 ml., incubated 3 hr. at 30° C. under 100% N_2.
[b] Values represent μl. CO_2 initially present.
[c] Values represent μl. CO_2 formed after 3 hr. of incubation.

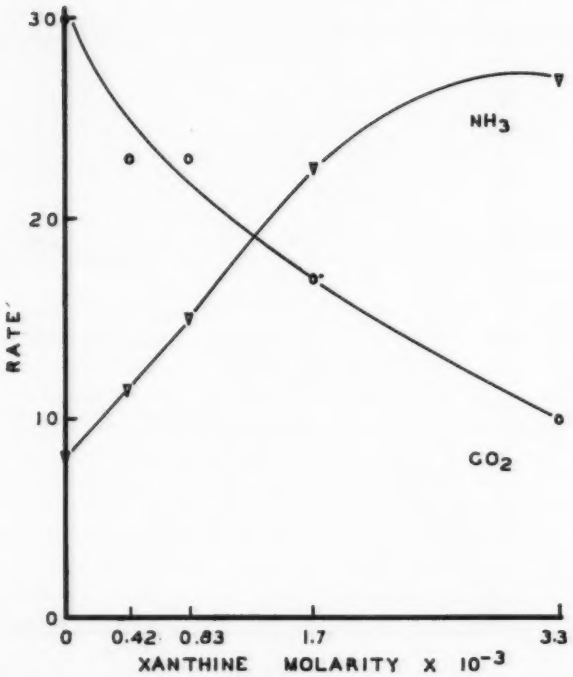

FIG. 2. Decarboxylation and deamination rates (expressed as μl. gases evolved/hr/mg N) from xanthine at various substrate levels (expressed as moles/liter $\times 10^{-3}$): incubation 3 hr. at 39° C. under 100% He.

dioxide evolution is obtained from xanthine in comparison to the endogenous. The fact that both methods (direct method *vs.* modified Dixon-Keilin) gave similar results indicates that sufficient bound carbon dioxide was present in the reacting system of the alkali-containing flask (direct method) to give reaction rates equivalent to those of the duplicate flask (without alkali).

Manometric decarboxylation rates were determined at various bacterial densities in the presence and absence of xanthine (Figure 4). Less carbon dioxide always was formed in the presence of this purine when compared to its corresponding endogenous value, at all bacterial densities. Since the initial carbon dioxide concentration increased directly with bacterial density (bacterial nitrogen content), the decarboxylation rate per mg. nitrogen of each point (Figure 4) was calculated and then plotted as a function of initial carbon dioxide concentration (Figure 5). From this graph, the endogenous decarboxylation rate decreases in a linear fashion as the carbon dioxide concentration increases. The reverse was true for the reaction observed in the presence of xanthine, because the decarboxylation rate is low at reduced carbon dioxide concentrations. However, the xanthine decarboxylation rate sharply

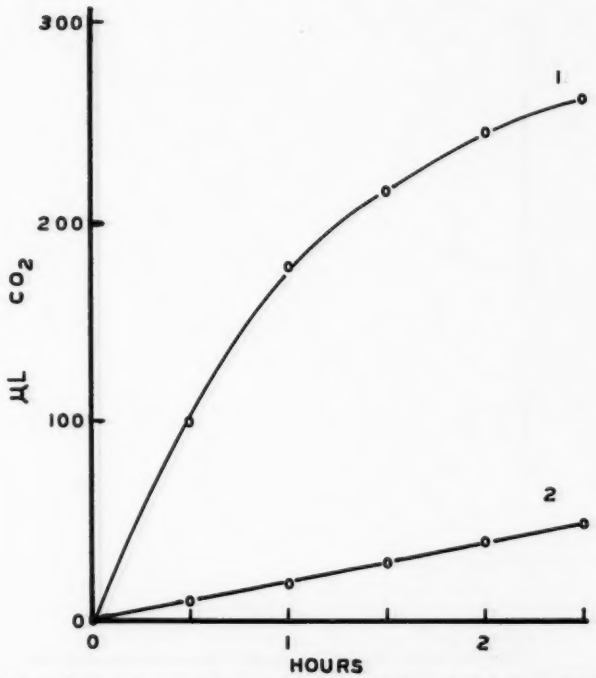

FIG. 3. Acid production (expressed as μl. CO_2 evolved) from 0.0017 *M* xanthine in 0.0015 *M* bicarbonate buffer: incubation 2.5 hr. at 39° C. under 1% CO_2 and 99% N_2: initial pH 7.1 (calculated): 1, xanthine; 2, endogenous.

increases, and approaches the endogenous value as the carbon dioxide concentration increases. In this same experiment, the production of hydrogen and/or methane was proportional to bacterial density (nitrogen) with both xanthine and WCS, and WCS alone (Figure 6). Slightly more hydrogen and/or methane was formed from this purine than from the endogenous, the rates being almost equivalent. This was not the case with carbon dioxide evolution. A direct proportionality seemed to exist between the formation of gases other than carbon dioxide and the amount of intracellular polysaccharide (ISS) present *(13)*. More hydrogen and/or methane were formed from cells containing larger quantities of ISS (determined qualitatively by iodine staining).

DISCUSSION

Theoretically, purines may have two possible fates in the rumen. They may be dissimilated by the rumen microflora and thereby utilized as a carbon or nitrogen source; or they may serve as growth factors for rumen bacteria. Studies employing the artificial rumen indicate that uric acid and allantoin are suitable nitrogen sources for the rumen microflora *(4)*. Other in vitro studies with mixed suspensions of rumen bacteria suggest that cellulose digestion im-

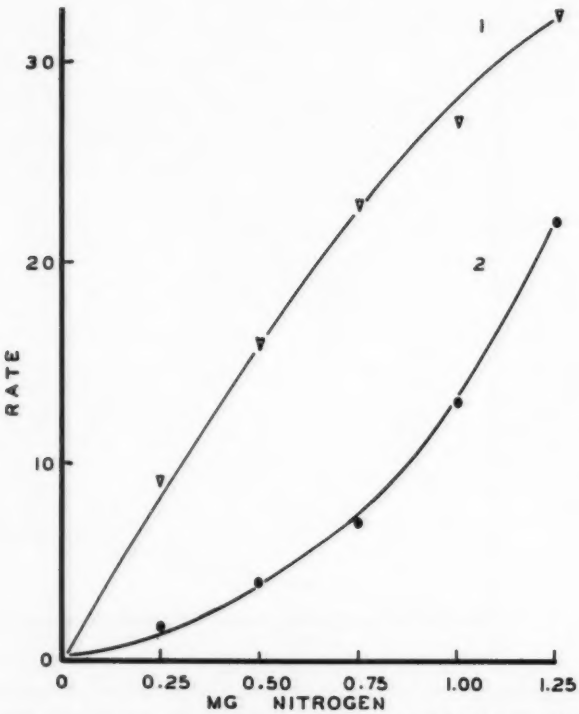

FIG. 4. Decarboxylation rates (expressed as μl. CO_2 evolved/hr.) from 0.0017 *M* xanthine at various bacterial densities (expressed as mg. *N*): incubation 2 hr. at 39° C. under 100% He : 1, endogenous; 2, xanthine.

proves considerably when purines are added to the reaction mixture *(5)*. In growth factor studies on the cellulolytic rumen bacterium, *Bacteriodes succinogenes*, purines and pyrimidines were used routinely in trace amounts, but the requirements of rumen microorganisms for these compounds have yet to be demonstrated *(7)*.

There are two general schemes for the anaerobic breakdown of purines *(21)*. Either the imidazole ring is split, yielding pyrimidine derivatives as intermediates as illustrated by *Micrococcus aerogenes (26)*, or the pyrimidine ring is split, yielding imidazole derivatives, i.e., as with *Clostridium acidiurici* and *Clostridium cylindrosporum*. In case of degradation by clostridia, the major end-products are ammonia, carbon dioxide, and acetic acid; whereas, with *M. aerogenes* these same end-products are encountered in addition to hydrogen, propionic and lactic acids, with trace amounts of pyrimidines. WCS of bovine rumen bacteria dissimilate purines, in vitro, using the same fermentative pattern observed with the nonrumen clostridial types. Other similarities are that both degrade xanthine, uric acid, and guanine hydrochloride completely, with

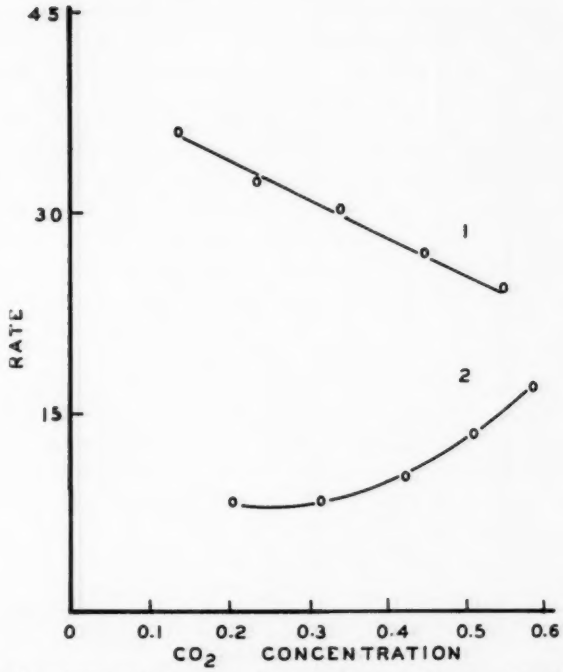

FIG. 5. Decarboxylation rates (expressed as μl. CO_2 evolved/hr/mg N) plotted as a function of CO_2 concentration (expressed as per cent initial CO_2 present per total volme): incubation 2 hr. at 39° C. under 100% He: 1, endogenous; 2, xanthine.

the formation of ammonia, carbon dioxide, and acetic acid; degrade hypoxanthine incompletely, and do not degrade adenine. This appears unusual, as it is known that anaerobic micrococci exist in large numbers *(15)* in the rumen, whereas clostridia rarely are encountered. If the micrococcal fermentation pattern was exhibited by WCS, then lactic, propionic acids, hydrogen, and/or methane would have been detected at some interval as end-products during in vitro dissimilation studies.

Purines are dissimilated by WCS at a slow rate, and deamination apparently proceeds after a prolonged incubation period, from 5 to 18 hr. This suggests that substrates as insoluble as purines require a prolonged reaction interval before significant dissimilation can be detected by biochemical assay of end-products. In this respect, in vitro dissimilation of purines may be compared to cellulose degradation *(8)*, as both are degraded by WCS after an incubation period of at least 24 hr. This further suggests that the rate of decomposition of any substrate is not indicative of its potential nutritive value in the rumen, because in most instances cellulose (insoluble carbohydrate) is the primary carbon source for ruminants. By analogy, the slow rate of attack on purines by WCS may not indicate its potential nutritional value. Deamination under

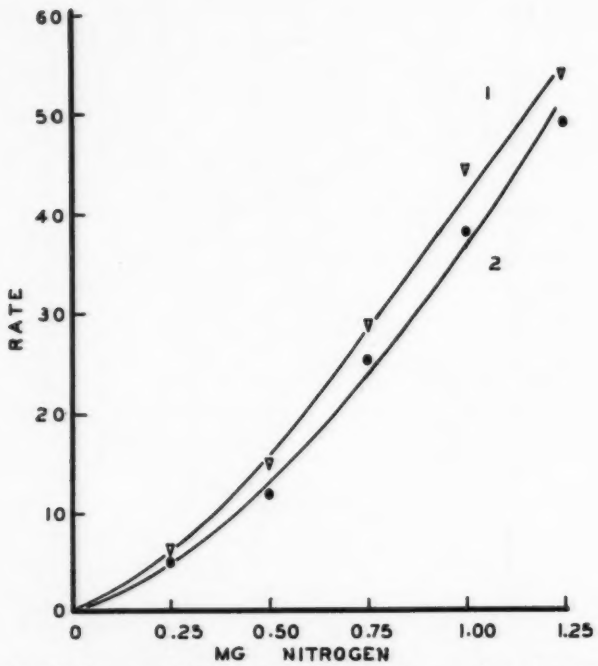

FIG. 6. Rate of hydrogen and/or methane production (expressed as μl. gases evolved/hr.) from 0.0017 *M* xanthine at various bacterial densities (expressed as mg. *N*): incubation 2 hr. at 38° C. under 100% He:1, xanthine; 2, endogenous.

alkaline conditions was greater than or equivalent to that observed at neutral pH values, excepting hypoxanthine. Increased solubility of purines at higher pH values may be a factor responsible for the increased deamination rate.

The activity of WCS on xanthine (as estimated by ammonia liberation, volatile fatty acid production, and residual purine determination) always was greater than that observed for guanine hydrochloride and uric acid. This would be expected if xanthine is the common intermediate in the degradation of all purines *(3)*. Constitutive enzymes are responsible for xanthine degradation, and adaptive enzymes are involved in the breakdown of uric acid and hypoxanthine. The inability to form adaptive enzymes may be due to the absence of these purines in the rumen or the inactivation of these purine-attacking enzymes during differential centrifugation procedures.

An indirect approach was used to determine whether carbon dioxide influenced reaction rates involved in xanthine degradation. Reaction rates were determined at various bacterial densities, using one substrate concentration level. In decreasing bacterial densities, carbon dioxide concentrations were decreased proportionally. (Carbon dioxide contamination from WCS is decreased at diminishing bacterial densities, until a critical carbon dioxide

concentration level is reached, at which point the reaction rate is not influenced.) Kinetic treatment of the data (Figures 4 and 5) indicates that the greatest differences in decarboxylation rates between xanthine and the endogenous is at minimal carbon dioxide concentrations, where the least amount of bacterial cells was present. At this level, the endogenous activity would be negligible and any carbon dioxide–utilizing response would be magnified. At high bacterial densities, endogenous decarboxylation reactions and contaminating bicarbonate of the mixed suspension are greater, and any carbon dioxide–utilizing response would be masked.

This type of effect was not observed with hydrogen and/or methane formation from the same series of experiments (Figure 6). The linear decrease observed in the endogenous decarboxylation rate at increasing carbon dioxide concentrations (Figure 5) suggests that a carbon dioxide–utilizing mechanism is normally present in WCS *(14)*. Carbon dioxide may act as the ultimate hydrogen acceptor forming methane, as demonstrated by McNeill *(20)* with WCS of rumen bacteria, or acetic acid as reported with *Clostridium aceticum (27)*. Carbon dioxide utilization during anaerobic purine dissimilation already has been established with clostridia *(1, 16)*. A similar finding is suggested in this study by indirect evidence: *(a)* xanthine is dissimilated readily in all long-term studies, yet carbon dioxide evolution from this purine always is smaller than the corresponding endogeneous value; *(b)* the xanthine decarboxylation rate decreases and the deamination rate increases as the xanthine concentration is increased (Figure 2); *(c)* acid production from xanthine always is greater than the corresponding endogenous value (Figure 3).

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TECHNICAL NOTES

EFFECT OF SODIUM CHLORIDE ON THE CURD TENSION OF RENNET-COAGULATED MILK

Among the soft pickled varieties of cheese which are usually made in warm climates, Domiati cheese is unique in being salted at the first stage of manufacture by adding it directly to the milk before renneting. The addition of an appreciable amount of salt to the milk, which sometimes reaches 15%, increases the time of coagulation and causes the formation of a softer curd *(3)*.

The study was undertaken, therefore, to determine quantitatively the effect of various concentrations of salt on the curd tension *(5)* of milk.

The investigations were carried out on cows' milk, both raw and pasteurized. In each case, the milk was warmed to 35° C. Samples of 100 ml. were placed in glass beakers 4.5 cm. in diameter and 6.5 cm. high. The beakers were then incubated in a water-bath regulated at 35 ± 0.5° C. One per cent of starter [either *Streptococcus faecalis (1)* or commercial *Streptococcus lactis*] was used in some of the pasteurized milk experiments. A ripening period of 1 hr. was allowed in this case. The required amount of salt was then dissolved in the milk. Standard Hansen's liquid rennet (diluted 1:20) was added at the rate of 1 ml. for each 10 lb. of milk. The rennet curd tension, after the appropriate time of coagulation, was measured by the curd-o-meter.[1]

The effects of rennet coagulation time and concentration of salt on the curd tension of raw milk are shown (Fig. 1). The curd tension is seen to increase with rennet coagulation time but to decrease with increasing salt concentration. It took 1 hr. and 45 min. for the milk containing 15% salt to reach the same curd tension value of 20 g. reached in 45 min. by the unsalted milk. In additional experiments with milk containing 15% salt, but not included in Fig. 1, it was found that the curd tension value had reached 85 g. at 3 hr. and 100 g. at 3 hr. and 30 min.

[1]Submarine Signal Co., Boston.

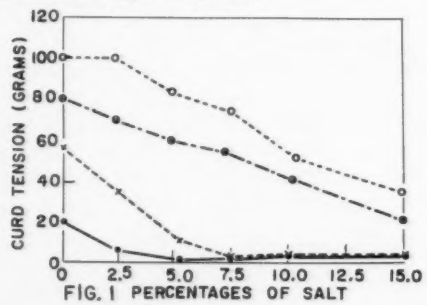

FIG. 1. Effects of salt and rennet coagulation period on the curd tension of raw milk.

Legend:

- ●———● Rennet coagulation period of 45 min.
- × - - - - × Rennet coagulation period of 1 hr. and 15 min.
- ⊙— - — - ⊙ Rennet coagulation period of 1 hr. and 45 min.
- ○ · · · · · · ○ Rennet coagulation period of 2 hr. and 15 min.

The addition of sodium chloride definitely reduced the curd tension of the milk for each coagulation period. Three possible explanations for this phenomenon have been presented by Fahmi and Sharara *(3)*. The reduction of the curd tension by the addition of other sodium salts has been reported by Tracy and Corbett *(9)*.

Figure 2 illustrates graphically the effect of salt and starters on the curd tension of ordinary pasteurized milk held 2 hr. and 15 min. for coagulation. Salt reduced the curd tension of pasteurized milk as it had for raw milk. The addition of 1% starter of the *S. faecalis*, and allowing 1 hr. ripening period before the addition of salt, definitely increased the firmness of the curd. In this case, the 2.5% salt did not reduce the curd tension; however, higher concentrations of salt did reduce the curd tension of milk. The addition of 1%

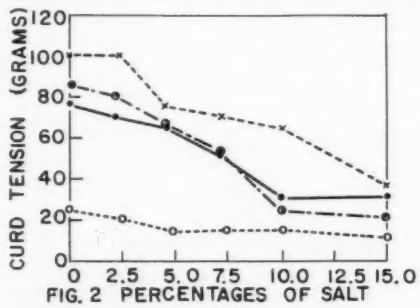

FIG. 2. Effects of salt and starters on the curd tension of pasteurized milk.

Legend:

- ●———● Pasteurized milk + salt and rennet.
- × - - - - × Pasteurized milk + 1% *S. faecalis* + 1 hr. ripening + salt + rennet.
- ⊙— - — - ⊙ Pasteurized milk + 1% *S. lactis* + 1 hr. ripening + salt + rennet.
- ○ · · · · · · ○ Homogenized milk + salt + rennet.

starter of the commercial *S. lactis* resulted in a slight increase in the curd tension of the unsalted milk and the milk containing 2.5% salt. No such effect, however, was observed for the higher concentrations of salt. The increase in curd tension of the milk containing starters (particularly the *S. faecalis* starter) may be due to the acid production by the organisms. The relationship between acid production and curd tension has been pointed out by Heinemann *(4)*. The fact that the curd tension of the samples containing the *S. lactis* starter does not increase for the higher salt concentrations, is probably due to the inhibition of the starter *(1)*.

The effect of the homogenizing process in decreasing the curd tension of milk has been shown by Doan and Welch *(2)* and by Theophilus, Hansen, and Spencer *(8)*. Sanders *et al. (6)* have shown the combined effect of homogenization and pasteurization in reducing the rennet curd tension of milk. Figure 2 shows that the combined effect of homogenization and pasteurization is more pronounced than the combined effect of pasteurization and salt. The addition of salt to the homogenized milk had a slight effect in reducing the curd tension of milk. At this lower level of curd tension, however, the sensitivity of the instrument may be reduced; therefore, the effect of salt may not be detectable.

In connection with the study of the manufacture of Domiati cheese *(7)*, an H_2O_2-catalase treatment was used for the purpose of reducing bacterial count. Therefore, it was considered desirable to determine if this treatment has any effect on the curd tension of milk. For this purpose, a sample of pasteurized milk and a sample of H_2O_2-catalase treated pasteurized milk were compared.

From the results shown (Table 1) it may be seen that the H_2O_2-catalase treatment had no effect on the curd tension of milk.

TABLE 1

Effect of H_2O_2-catalase treatment on the curd tension of milk

Treatment	Time of coagulation 1 hr.	2 hr.	3 hr.
Pasteurized milk + 7.5% salt + rennet	25	50	60
Pasteurized milk + H_2O_2-catalase + 7.5% salt + rennet	25	50	65

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PRELIMINARY RESULTS ON THE PRESERVATION OF BOVINE SEMEN IN A MILK-EGG YOLK-GLYCEROL EXTENDER[1]

The findings of McLean *(3)*, that the addition of glycerol to either egg yolk citrate or boiled homogenized whole milk diluters would prolong the livability of bovine semen, has prompted the rather widespread use of glycerolated extenders for liquid semen. Flipse and Almquist *(2)* found that glycerol at the 5.0% level gave significantly higher spermatozoan motilities in stored semen than did 2.5% glycerol, when each was added to an egg yolk–glycine diluter. More recently, Almquist *(1)* has reported on the use of a milk-glycerol ex-

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 1792. Approved by the Director.

tender in which the fertility of bovine spermatozoa was satisfactorily maintained for at least four days following collection. VanDemark is reported *(4)* to have found slightly higher conception rates with spermatozoa suspended in a combination of unheated, pasteurized skimmilk, 7:3 yolk-citrate, as contrasted to spermatozoa suspended in a yolk-citrate extender.

Work in progress at the University of Missouri indicates that the use of a combination of heated skimmilk, egg yolk, and glycerol will maintain spermatozoan motility longer than heated skimmilk and glycerol or egg yolk citrate and glycerol. The average of the results for six split-sample storage trials for semen from four different bulls is shown (Figure 1). Postdilution spermatozoan motilities for all samples

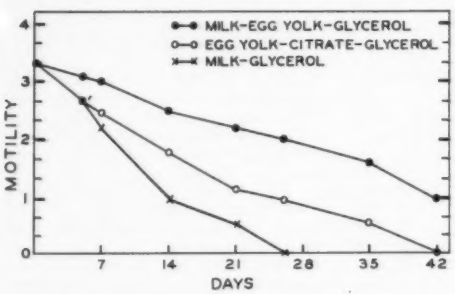

FIG. 1. Postdilution motility of bovine semen stored at from 0 to −3° C. in various glycerolated extenders.

averaged 3 + (0 − 5 motility scale) on the day of collection. Time required for motility to decline to a rating of two (minimum motility rating for semen used in University of Missouri herds) was 8, 12, and 25 days, respectively, for semen stored in heated skimmilk + glycerol, egg yolk citrate + glycerol, and heated skimmilk with egg yolk and glycerol. Progressively motile spermatozoa have been observed in some of the latter samples after 74 days of storage. The diluent was prepared by adding egg yolk (10% by volume) to skimmilk that had been heated (to 95° C.) and cooled. Immediately after collection, semen was diluted to one-half the desired final volume with the milk–egg yolk mixture. The semen-milk-egg yolk mixture was then cooled gradually to 5° C. before final dilution with milk–egg yolk diluent containing 16% glycerol. The glycerolated extender was added in four aliquots at 30-min. intervals. The diluted semen containing 8% glycerol was then placed in 1 cc. plastic vials (vials should be completely filled to minimize exposure to air). The vials were sealed and put in a water bath (5° C.) which, in turn, was placed in a refrigerator for storage at from 0 to −3° C. Freezing point of the extended semen was approximately −5° C., thus making possible liquid semen storage in ice bath.

Preliminary results indicate that the optimum extender composition is approximately 82% skimmilk, 10% egg yolk, and 8% glycerol.

Further studies are in progress on the fertility of semen stored in the milk-egg yolk-glycerol extender and on the factors responsible for the apparent synergistic action of skimmilk and egg yolk constituents in the presence of glycerol.

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FREEZE-DRYING BOVINE SPERMATOZOA

Lyophilization is being used for preserving blood plasma, antibiotics, milk, bacteria, tissue culture media, viruses, and various metazoan cells. Neumann *(4)* found that live tumor cells would withstand freeze-drying. Passey and Dmochowski *(5)* had reported earlier that living tumor cells could be observed only after partial drying. The literature on freeze-drying of spermatozoa of all species is limited. Polge *et al. (6)*, using a high-vacuum distillation process on fowl semen, recovered motile spermatozoa. Sherman *(7)* observed negative results with human spermatozoa, when using freeze-drying procedures commonly employed in preparation of materials for cytological studies.

A freeze-drying study was conducted using five bulls (three Holsteins, one Guernsey, and one Jersey). Eight samples were collected and extended 1:4 at 95° F. into whole milk containing 1,000 μg. of streptomycin/ml of extended semen. The extended semen was then

placed in a 400-ml. beaker filled one-third with water at 85° F. and allowed to cool for 20 min. (to approximately 65° F.) at 40° F. Then the extended semen was removed from the water bath and allowed to cool at an air temperature of 40° F. for 1 hr. Commencing at 80 min. postcollection, whole milk extender containing 20% glycerol was added in one-third quantities every 20 min. The semen then had a final extension of 1:8 and contained 10% glycerol per ml. of extended semen. Six of the glycerolated-extended semen samples were allowed to equilibrate for 18 hr. and the remaining two for 10 hr. at 40° F.

After the equilibration period, 1/8-ml. aliquots of glycerolated semen were placed into glass vials suitable for proper shell-freezing (5 ml. tear-drying bulbs). The samples were placed into an inner ethanol freezing bath at 40° F., with the outer isopropanol bath at 30° F. The vials of semen were frozen at the rate of 2.5° F/min to 5° F. Large amounts of dry ice were then added to the inner container, to insure a rapid drop in temperature. When the temperature of the semen reached −60° F., the vials were attached to a Virtis Macro Freeze-Drying Unit. The drying process required approximately 45 min. The vials were removed from the lyophilizer and stored at −110° F. for approximately 10 min. The lyophilized extended semen was restored to original volume by rapid addition of a 3% fructose solution at 100° F. Reconstitution was immediate.

Approximately 5-10% of the spermatozoa in the first five samples of semen from five different bulls showed progressive motility after reconstitution. Though only a small percentage of the spermatozoa recovered their motility, those which did showed very rapid and normal movement. This result suggests that a major portion of the loss in motility was due to physical hazards created during freezing and subsequent desiccation. Spermatozoa in three later samples did not survive, and in each case there was escape of vacuum from the Virtis Lifilizer as the frozen semen-glycerol-whole milk samples were being attached. Also, two of these three samples had been equilibrated for only 10 hr.

Mixner and Saroff *(3)* have found that levels of glycerol above 4% increase the permeability of the living and motile sperm cells, allowing some of them to be counted as dead cells in the differential staining procedure. Therefore, differential staining procedures were not attempted.

The amount of water removed during the freeze-drying process in this study was not calculated. However, Polge *et al. (6)* have estimated 90% of the original water was removed when a distillation process of high vacuum against liquid air was used to desiccate fowl semen, diluted with 30% glycerol in Ringer's solution. From limited data, Polge *et al.* observed up to 30% motile spermatozoa following reconstitution. Bialy and Smith *(1)*, using a variety of techniques in freeze-drying bovine semen, observed negative results when dehydration level exceeded 71%. Leidl *(2)* observed living bull spermatozoa following freeze-drying and reconstitution, when the final water content did not go lower than 5% and the glycerol content did not exceed 50%. He observed no motile sperm unless reconstitution was made immediately after completion of the freeze-drying process.

From these limited data, and those of Leidl, it appears certain that bovine spermatozoa can survive freezing and subsequent lyophilization, as evidenced from recovery of limited numbers of progressively motile sperm.

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OUR INDUSTRY TODAY

MANUFACTURE AND TECHNOLOGY OF SWISS CHEESE [1]

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Swiss cheese, which originated in Switzerland, is often referred to as the King of Cheeses. In addition to Switzerland, the countries which are prominent in the manufacture of this cheese include the United States, Finland, and Denmark. In its home country, the cheese is known as Emmentaler cheese; in other countries, as Swiss cheese. Genuine Swiss cheese has a flavor and aroma which cheese-makers in other countries have had difficulty in duplicating. This superiority has generally been credited by cheese-makers to the aroma of certain alpine grasses, which is imparted to the milk and then becomes a part of the cheese flavor. But exact duplication of all of the many variables involved in manufacturing and ripening probably would result in Swiss cheese comparable in flavor to that of Emmentaler.

Characteristics of Swiss Cheese

Swiss cheese, as defined in the Federal Register, is the food prepared from cow's milk by the so-called Swiss Cheese Procedure. It has holes or eyes developed throughout the cheese, contains not more than 41% moisture and not less than 43% milk fat in the dry matter, and is not less than 60 days old.

Four grades are recognized in the United States Standards for Swiss cheese. Grade A Swiss is characterized by being free from off-flavors. In addition, it should possess some degree of characteristic Swiss cheese flavor, depending on its age. The body should be uniform, firm, and smooth. The eyes should be round or oval and uniformly distributed, with the majority not being over ½ in. in diameter. A plug of cheese taken with a No. 8 trier should show from one to eight eyes. The color should be uniform and the rind should be dry and free from defects.

Swiss cheese of Grades B, C, and D quality deviates from Grade A cheese in one or more of these standards. The greater the deviation, the lower the grade of the cheese.

Down-grading of Swiss cheese usually results from the use of inferior milk, or because of improper acid development during making. Such conditions may result in cheese with a spongy or gassy body and faulty eye development. Over-set, rough-eyes, dead-eyes, and blind are some of the terms used to describe abnormal eye formation. Off-flavors encountered in defective Swiss cheese may include fermentation flavors such as fruity, sour, and yeasty. Flavors such as weed and onion can be attributed to feeding conditions.

Manufacturing Procedure

Swiss cheese is traditionally manufactured in copper kettles. This is in contrast to other types of cheese, which are manufactured in vats of more inert materials, such as tin-plated metal or stainless steel. A kettle may contain from 2,500 to 3,500 lb. of milk, sufficient to make one Swiss cheese ranging in weight from 160 to 250 lb.

Milk with a methylene-blue reduction time from 4 to 5 hr. is usually satisfactory for the manufacture of Swiss cheese. The milk is partly skimmed, because whole milk often results in cheese of inferior body and texture characteristics, causing the defect termed as glass. Raw milk is used by most factories, although milk heated to less than pasteurization temperature is used advantageously in many places. Attempts to make Swiss cheese from completely pasteurized milk have not been entirely successful.

The following is a schematic outline of the steps generally involved in the manufacture of Swiss cheese. Slight adjustments in the procedure may be necessitated by the quality of the milk, the cultures used, and the season of the year.

Addition of water to the milk before setting is a common procedure in Europe, but is not practiced in this country. The procedure results in a cheese with a more elastic body, because of the dilution of the lactose and a subsequent reduction in lactic acid formation. However, it requires the best of milk and a pure water supply.

The recommended amounts of starters pertain to milk-starters. Almost twice these amounts are required when whey-starters are used. The cultures are added before the rennet, and the milk is frequently allowed to ripen for about 30 min., because most cheese-makers want slight acid development before adding rennet.

The rather high setting temperature makes it necessary for the cheese-maker to watch the curd closely, to determine the proper time of harping (cutting). If harping is delayed, it

[1] Article No. 13-57. Department of Dairy Technology, The Ohio State University.

Schematic Outline of Procedure for Manufacture of Swiss Cheese

Cultures
Rod—0.05–0.1%
Coccus—0.05–0.1%
Propionibacterium—1 ml/100 lb. milk

Water	0–5%
Rennet	2–3 oz/1,000 lb. milk
Setting temperature	90°–92° F.
Setting time	25–30 min.
Cutting (harping)	10–15 min.
Foreworking	20–30 min.
Heating temperature	122°–124° F.
Cooking	30–40 min.
Stirring out	30–40 min.
Turned on press, first day	4 times
Dry salt	24 hr.
Brinesalt	48–72 hr., 54–56° F., 22% brine
Cold curing room, 56° F.	12–14 days
Warm curing room, 68–74° F.	5–7 wk.
Cold curing, 54–56° F.	Until sold

—Courtesy Town Hall Cheese Co., Wis.

FIG. 1. Hooping of wheel style of Swiss cheese, showing texture of freshly dipped curd.

may be impossible to get a fine curd. This may result in high-moisture cheese. Heating should be conducted with care. Rapid heating may result in formation of a film on the curd particles which would retard, or prevent, proper expulsion of whey.

Temperatures lower than 122-124° F. prolong the cooking time and bring about too rapid acid development in the vat. Temperatures above 122-124° F. adversely affect the acid-producing bacteria. It has been demonstrated experimentally that the higher the temperature of cooking over 124° F., the poorer the quality of the cheese. The major criticism of such cheese was excessive eye-formation.

The firmness of the curd before hooping determines the moisture content and the amount of acid which will be formed in the cheese, thereby establishing the body and texture characteristics of the cheese (Figure 1).

The dipping process consists of removing all of the curd from the vat in one mass. Care must be taken to insure a compact mass of curd in the hoop. If the interior is broken, a whey-pocket may result. Such a pocket is almost impossible to press out, and eventually abnormal fermentations will occur.

Pressure should be applied slowly to the cheese on the press, to allow whey to escape. When turning the cheese, care should be taken not to tear the surface of the cheese if the cloths stick.

Salting and curing to some degree determine the appearance and quality of the cheese. Some cheese-makers favor a slight dry-salting for 24 hr. before placing the cheese in the brine; whereas, many use only brine salting. The use of dry salting results in a better rind formation. Salt is inhibitory to eye-formers, which makes it possible to control these bacteria by varying the final salt concentration in the cheese.

After the cheese has been salted, it is allowed to dry for a day and is then moved to a cold room, where it is placed on shelves on circular boards. The temperature in the cold room ranges from 50 to 54° F. Salt is sprinkled on the surface of the cheese two or three times a week and the surface is then scrubbed and wiped off, to prevent surface growth (Fig. 4).

After about 2 wk. at this temperature, or when the texture of the cheese has become sufficiently elastic to support eye-formation, the cheese is transferred to storage at from 68 to 74° F. The treatment of the surface is continued, because only little surface growth is desirable until the cheese is about 6 wk. old. The propionibacteria grow in the warm room and eye formation results. After about 5 wk., eye formation usually is complete. This can be tested with a trier and by observing the appearance of the cheese. The cheese is then moved to a cold room at about 52° F., where curing is completed.

The curing room should be kept at from 85 to 90% relative humidity, to prevent excessive moisture loss. The cheese should be turned twice a week to prevent rind rot.

Modifications of the Swiss Cheese Manufacturing Process

A relatively recent development, which has revolutionized the entire Swiss cheese industry in the United States, is the introduction of rindless block Swiss cheese. Two principal methods

for the manufacture of this type of cheese are employed.

One method utilizes stainless steel vats of up to 20,000-lb. capacity for making the curd, rather than the conventional copper kettles. When the curd is sufficiently firm, the whey and curd are pumped to a special device which serves as strainer and hoop, where the curd is collected. The curd is then pressed and the large block of cheese is cut into smaller blocks of more convenient size. After the cheese has been salted, the surface of the cheese is dried. The protection of the surface is afforded by placing the cheese in a large plastic bag. The air is evacuated from the bag, the bag is heat-sealed, and the entire unit dipped in cheese-wax as an added precaution against air leaks.

The second method of manufacture of rindless block Swiss cheese calls for the cheese being made in the conventional copper kettle. After the curd is firmed sufficiently, the curd mass is transferred to a square hoop and pressed, (Figure 2). The next morning, the

FIG. 2. Pressing of block-style Swiss cheese.

square cheese is cut into two pieces and brine salted. After salting, the surface of the cheese is dried and a thin layer of petrolatum applied. The final protection of the cheese surface is brought about by wrapping the cheese in a piece of plastic material, which is sealed by tape only.

After wrapping, rindless block Swiss cheese is placed in wooden or carboard boxes which are large enough to allow for upward expansion of the cheese. Curing takes place as for regular wheel Swiss, with occasional turning being the only treatment needed.

Rindless block Swiss cheese has definite economic advantages over the conventional wheel cheese, in regard to handling and merchandising. Whereas the appearance of the cut cheese is little different from that of the wheel Swiss, the flavor which develops in the wrapped cheese often is lacking, compared to that of regular Swiss.

Technology and Science of Swiss Cheese Manufacture

Cultures. Three different bacteria are used as cultures in Swiss cheese making: *Streptococcus thermophilus* (the coccus), *Lactobacillus bulgaricus* (the rod), and *Propionibacterium shermanii* (the eye former).

S. thermophilus and *L. bulgaricus* are generally selected on their acid-producing capacity. Little or no attention is given to the contribution they may make to flavor development. *S. thermophilus* is responsible for the acid produced during the first 7 to 8 hr., and *L. bulgaricus* is responsible for the acid developed from 8 to 21 hr. After about 21 hr., acid development in the cheese is completed.

The role of *S. thermophilus* during the ripening process is not entirely understood, whereas the role of *L. bulgaricus* is assumed to be the break-down of casein.

Proper care of cultures, to maintain actively growing strains and to avoid contamination, is an important part of Swiss cheese manufacture. Mother cultures of *S. thermophilus* and *L. bulgaricus* should be propagated every day. Some cheese-makers prefer to carry the cultures in milk, others prefer whey. Whey has some advantage, because it contains certain growth-promoting factors developed during cheese manufacture. However, when whey is used, great care must be exercised to assure destruction of bacteriophage by sterilization. The medium should be autoclaved for 15 min. at 15 lb. pressure, or at least heated by steam or water to approximately 200° F. for 1 hr. For best results, the medium should be made fresh every day. After it has been heated, the milk or whey is cooled to 98-100° F., inoculated with 0.1% culture, and placed in an incubator at 98-100° F.

Milk may be used as the medium for bulk starter and should then be heated to 190° F. for 1 hr. and cooled before inoculation. Frequently, however, whey is used for this purpose. The whey is pasteurized at 150-160° F. for 30 min., cooled and inoculated with the acid-formers, either singly or in combination. Bulk starters should also be incubated at 98-100° F.

An incubation time of 16-18 hr. is usually satisfactory. Milk cultures of Lactobacilli should then have an acidity of 1.1-1.25%, and milk cultures of Streptococci should have an acidity of 0.75-0.80%. Whey cultures will usually have about 0.4% less acid than milk cultures.

After incubation, mother cultures should be cooled to 55-50° F. until transferred. Bulk starters should be used within a few hours. Prolonged incubation results in a decrease

in the rate of acid formation which could affect the quality of the cheese.

P. shermanii may be carried in a simple medium of 1% lactate, plus 1% yeast extract, in distilled water adjusted to pH 6.6. Media containing whey or whey powder may also be used. In general, media containing some lactate are better than media containing only lactose as the major source of carbon, because these bacteria do not readily ferment lactose. The medium should be autoclaved, as described previously for milk. After inoculation, the cultures are incubated at about 86° F. for from two to three days in lactate media, or from five to seven days in lactose media. These cultures remain viable for weeks and do not require daily transferring.

Changes During Ripening

Experience has demonstrated that the probability of getting the best flavor developed in Swiss cheese is when the pH of the cheese reaches 5.16 to 5.25 within 14-16 hr. after addition of rennet. Deviations from this usually result in cheese with poor eye formation and flavor. As the cheese ripens, pH increases and reaches a value of from 5.70 to 5.80 after about 6 mo. of curing. The increase is rather uniform throughout the ripening period.

The fermentation of part of the lactate to propionate and acetate, and the degradation of proteins which takes place during curing, may be contributing factors to the increase in pH.

Propionic acid bacteria are generally credited with the fermentation of lactate, with flavor and eyes developing concurrently. The over-all fermentation involves:

$$3\ CH_3CHOHOO^- \longrightarrow 2\ CH_3CH_2COO^- +$$
Lactate — Propionate

$$CH_3COO^- + CO_2 + H_2O$$
Acetate — Carbon Dioxide — Water

The exact mechanism of the reaction is still debated. The carbon dioxide formed during the fermentation accumulates in weak spots in the cheese and forms the eyes.

That propionic acid bacteria are not responsible for all of the carbon dioxide present in Swiss cheese was recognized as early as 1912 by Clark *(2)*. He found that the concentration of carbon dioxide in Emmental cheese was much higher than could be explained by the propionic acid fermentation alone. He distinguished between a saturating and an inflating gas production. The former was presumed to arise from carbohydrate fermentation by lactic acid bacteria and to be equally as important to eye formation as the latter, arising from the propionic acid fermentation.

Recent research in this department has demonstrated that a bacterial fermentation system related to the citric acid cycle is functioning in Swiss cheese. One of the products of this cycle would be carbon dioxide, thus confirming Clark's findings.

Theoretically, the propionic acid fermentation should yield propionic and acetic acid in a ratio of 2:1. Such a ratio rarely, if ever, exists in Swiss cheese. The ratios encountered in this laboratory have ranged from 1.6:1 to 0.5:1, with ratios of 1.5:1 to 1.2:1 being more common.

Succinic acid has been demonstrated to be one of the products of the propionic acid fermentation, but the concentrations demonstrated in Swiss cheese have not been sufficient to account for the low ratios of propionic to acetic acid.

A more likely explanation for the low ratios could be that acetic acid originates from other reactions in addition to the propionic acid fermentation. Decarboxylation of pyruvic acid, which is present in Swiss cheese in higher concentrations than in any other variety, would yield acetic acid. The amino acid glycine would yield acetic acid if deaminated.

In addition to propionic and acetic acid, Swiss cheese also contains butyric and higher volatile fatty acids, including valeric acid. Milk fat, carbohydrate, and amino acids all have been suggested as possible sources of these fatty acids. The role of these acids is not too well understood, although it has been established that excessive concentrations of butyric acid are associated with inferior cheese.

With the advent of comparatively simple but highly accurate methods for the separation and quantitative determination of free fatty and amino acids, it was discovered that propionic and acetic acid are not entirely responsible for the characteristic flavor in Swiss cheese. Analyses demonstrated that cheeses with high and low flavor intensities could have identical levels of these acids. Correlation of propionic acid contents with free amino acid contents showed that high concentrations of the amino acid proline and/or the conditions leading to high concentrations of proline were as essential to characteristic flavor as high levels of propionic acid. Aged Swiss cheeses lacking in flavor were low either in proline or in propionic acid, indicating the importance of a proper balance of both of these compounds for characteristic flavor.

Analysis of wheel and rindless block Swiss cheese has contributed further evidence to the role of a high concentration of proline to the development of characteristic Swiss cheese flavor. Wheel Swiss contained several times as much proline as did rindless cheese. The two types of cheese contained approximately the same concentrations of propionic and acetic acids. The wheel Swiss cheese was considered typical in flavor; whereas, the rindless was criticized for being lacking or atypical and putrid. The rindless block Swiss cheese included

in this study was made in copper kettles and the plastic wrapper sealed with tape only.

The occurrence of free amino acids is caused partly by break-down of casein by *L. bulgaricus* and perhaps also by *S. thermophilus*. Some free amino acids may originate through transamination, involving glutamic acid and keto acids from carbohydrate or citric acid fermentation. The contribution of propionibacteria to the amino acid pattern of Swiss cheese has not been established. That this bacterium in some manner is responsible for the high levels of proline, either singly or in symbiosis with the two lactic acid cultures, cannot be excluded. Other cheese varieties, where *S. thermophilus* and *L. bulgaricus* are used as acid-producers, are not characterized by high levels of proline.

Besides the previously mentioned compounds, a number of trace compounds are associated with the flavor of Swiss cheese. This contribution is either indirect, the compounds being intermediary in fermentation cycles, or direct, the compounds being end-products of fermentation. The exact status of many of these compounds is difficult to establish. Perhaps it is safe to assume that all compounds present contribute to the flavor of cheese, whether they be considered intermediary or end-products.

The more prominent of the trace compounds identified in Swiss cheese include pyruvic acid and members of the previously mentioned citric acid cycle. The member compounds include oxalsuccinic, oxalacetic, alpha-ketoglutaric, glyoxylic, and alpha-acetolactic acids.

The presence of these compounds in fresh cheese can be explained on the basis of the fermentation of citrate. The increases in concentration of these compounds during curing are not so readily explained, since citrate presumably is exhausted during the first few weeks of ripening. Keto acids which are derived from amino acids and fatty acids in other varieties of cheese are generally absent in Swiss cheese. However, iso-keto-caproic acid which could originate from iso-leucine has been isolated from some Swiss cheeses.

In addition to the acidic carbonyl compounds, a number of neutral carbonyl compounds are present in Swiss cheese. Some have been identified, such as diacetyl, acetylmethylcarbinol, and acetone. A number of longer chain compounds present have not yet been identified.

The major fermentations characterizing Swiss cheese are shown (Figure 3).

Role of Copper in Swiss Cheese

Swiss cheese is the only variety of cheese traditionally manufactured in copper vats, exclusive of a few very closely related varieties. All other varieties are made in vats having a relatively inert surface, such as tin or stainless steel, as previously mentioned. With the advent of stainless steel for the manufacture of dairy equipment, attempts were made to make Swiss in stainless steel vats. So far, these attempts have met with mixed success. The flavor and body characteristics of Swiss cheese made in stainless steel vats generally are considered inferior to those of Swiss cheese made in copper kettles.

Swiss cheese made in copper kettles contains about 17 p.p.m. of copper. The presence of as little as 3 to 4 p.p.m. of copper is usually detrimental to other varieties of cheese. The body and texture of Swiss cheese are quite different from those of other hard varieties.

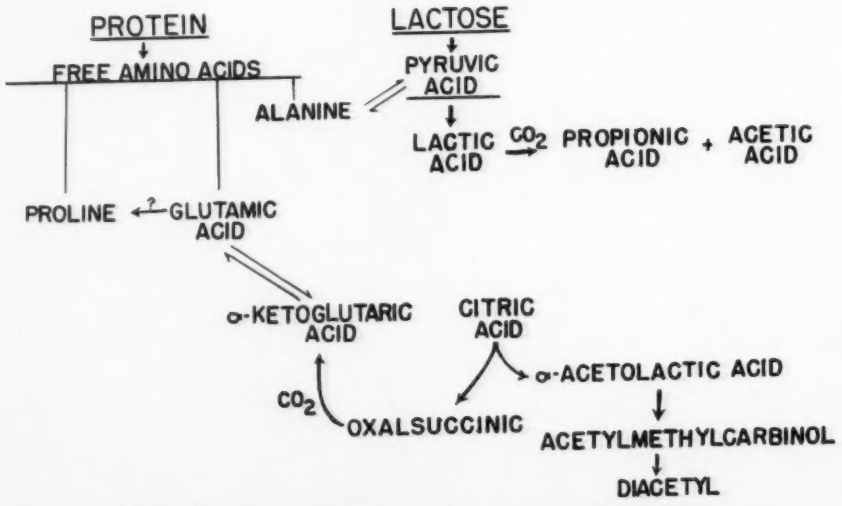

FIG. 3. Major fermentation pathways in the ripening of Swiss cheese.

To a large extent this can be attributed to differences in manufacturing procedure and cultures, but copper, perhaps by combining with protein, probably plays a role in bringing about the desired characteristics. At least, Swiss cheese made in stainless steel vats often has body and texture characteristics different from Swiss made in copper kettles, and does not support proper eye formation. "Slits" rather than eyes often occur in cheese made in stainless steel kettles.

The effect of copper on flavor development may be indirect. High-grade Swiss cheese, more than any other cured variety, is void of proteolytic flavor. From studies on milk, it is known that copper reacts with sulfhydryl groups, and greatly reduces the number of these groups exposed by subsequent heat treatment. The role of copper in Swiss cheese may be similar—to react with sulfhydryl groups in such a manner as to prevent extensive exposure of active sulfhydryl groups and release of hydrogen sulfide during ripening.

Recent work in Switzerland *(1)* indicated that proteolysis during ripening was more extensive in Swiss cheese made in stainless steel vats than in cheese made in copper kettles; however, the amount of soluble nitrogen formed was largest in the Swiss cheese made in copper kettles. Also, the propionic acid fermentation appeared to be affected adversely in cheese made in stainless steel vats, particularly in the later stages of ripening. These results could indicate that in Swiss cheese made in stainless steel vats, the exposure of more sulfhydryl groups, with the possible release of more hydrogen sulfide, interferes with the propionic acid fermentation.

FIG. 4. Surface care of wheel-type Swiss cheese in the warm curing room.

Summary

The successful outcome of Swiss cheese manufacture is dependent on a number of factors. Acid-forming and eye-forming bacteria have to be selected and properly taken care of by frequent propagations. Every step involved in the manufacturing procedure must be applied in a manner which will insure a proper rate and amount of acid development.

During curing, degradation of the constituents of the cheese occurs. Fatty acids and amino acids are liberated. Certain compounds important in intermediate bacterial metabolism also are formed.

Characteristic flavor and body and texture development of Swiss cheese appear to be dependent on a proper balance of products of metabolism. Poor milk, improper rate of acid development, and adverse curing-room conditions are some of the causes of low-quality Swiss cheese. Certain theories are advanced regarding flavor development and the role of copper in Swiss cheese.

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OUR ASSOCIATION

SCORE-CARD AND GUIDE FOR COTTAGE CHEESE[1]

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The scoring guide enumerates the common defects and the suggested score. This guide should assist in standardizing the grading of cottage cheese for contests or for routine plant quality control. The suggested ranges for the defects are based largely on 20-yr. averages of judges' scores of other dairy products at the International Students' Contests. The terms which have been used are defined, in order to assist in describing the defects. The product is considered unsalable if it is scored below the normal range.

[1]Published according to the policy stated, *J. Dairy Sci.*, **40**: 1369. 1957.

Ideal cottage cheese. The flavor of cottage cheese should be similar to fresh, clean skimmilk or cream (if creamed). Cottage cheese should have a slightly acid, salty taste, with the delicate flavor and aroma of a good lactic starter (diacetyl). The body of cottage cheese should be uniform, smooth, and meaty (not too firm or too soft and pasty). The curd particles

Scoring guide for cottage cheese[a]

Flavor defects	Slight	Distinct	Strong	Excellent 45–40 No criticisms	Good 39.5–38.5	Fair 38–36.5	Poor 36–32	Bad 31.5–0
Flat	39.5	39	38.5	Normal	Sl.[b] D.S.			
Feed	39	38.5	37.5	Range	Sl.D.	S.		
High salt	38.5	38	37	42–32	Sl.	D.S.		
Acid	38.5	38	36		Sl.	D.	S.	
Storage	38	36	34			Sl.	D.S.	
Sour	37	34	32			Sl.	D.S.	
Medicinal	37	34	31			Sl.	D.	S.
Foreign	37	34	31			Sl.	D.	S.
Stale	36.5	32	29			Sl.	D.	S.
Bitter	36	32	28				Sl.D.	S.
Unclean	36	31	27				Sl.	D.S.
Malty	35.5	30	25				Sl.	D.S.
Metallic	35	29	24				Sl.	D.S.
Fruity or fermented	35	28	23				Sl.	D.S.
Musty	34	27	22				Sl.	D.S.
Oxidized	33.5	25	18				Sl.	D.S.
Yeasty	33	24	15				Sl.	D.S.
Rancid	32	23	14				Sl.	D.S.

Body and texture defects	Slight	Distinct	Strong	No criticisms Excellent 35–34.5	Good 34–32.5	Fair 32–30	Poor 29.5–0
Firm or hard	34	33	32	Normal	Sl.D.	S.	
Weak	33.5	32.5	31.5	range	Sl.D.	S.	
Mealy	33	32	31	34.5–31.0	Sl.	D.S.	
Rubbery	32.5	31.5	30		Sl.	D.S.	
Pasty	32	31	28			Sl.D.	S.
Gelatinous	31.5	29	26			Sl.	D.S.

Color and appearance defects	Slight	Distinct	Strong	Excellent 15–14.5 No criticisms	Good 14–13	Fair 12.5–11	Poor 10.5–0
Lacks uniformity	14.5	13.5	12.5	Sl.	D.S.		
Overcreamed	14	13	12	Normal	Sl.D.	S.	
Lacks cream	13.5	12.5	11.5	range	Sl.	D.S.	
Shattered curd	13	12	11	14.5–11.0	Sl.	D.S.	
Matted	13	12	11		Sl.	D.S.	
Unnatural color	12.5	11.5	10			Sl.D.	S.
Free whey	12	11	9			Sl.D.	S.
Translucence	11.5	10	8			Sl.	D.S.
Surface discoloration	11	9	7			Sl.	D.S.
Slimy	10	8	5				Sl.D.S.

[a]Defects listed in order of most objectionable.
[b]Sl.—Slight (barely detectable), D.—Distinct, S.—Strong.

should be discrete, uniform in size (regardless of style), and of a natural, creamy-white color. Creamed cottage cheese should have a uniform layer of cream around the curd particles, with a minimum of free cream. Any excess cream should be of a thick consistency (not whey-like or watery).

TERMS USED TO SCORE COTTAGE CHEESE

Flavor—45—Normal Range, 42–32

Bitter—An astringent, puckery taste.
Feed or weed—The aromatic flavor characteristic of certain feeds and weeds.
Flat—Lack of characteristic flavor.
Foreign—Any objectionable flavor not characteristic of the product and not specifically listed.
Fruity or fermented—The aromatic flavor of orange, pineapple, strawberry, etc., usually due to bacterial action.
High acid—An excess acid over and above that considered desirable, but which is clean and sharp.
High salt—Excessive salt over that desired by consumer.
Malty—A malt-like flavor due to bacterial action.
Medicinal-Chemical—The smell and taste of disinfectant, chlorine, iodine, or phenolic compounds.
Metallic—A metal taste. Astringent. (A similar taste is noted when a penny is placed in the mouth.)
Musty—The odor of a damp, poorly ventilated cellar, or refrigerator.
Oxidized—A cardboard flavor.
Rancid—The pungent, rasping aroma and taste of butyric acid.
Sour—Excessive acid usually associated with other bacterial defects, such as fruity, fermented, etc., and is not clean.
Stale—A lack of freshness.
Storage—Odors absorbed from storage rooms or refrigerators.
Unclean—Reminiscent of the odor of dirty equipment, possibly due to inferior milk.
Yeasty—The aromatic flavor, characteristic of any fermentation by yeasts.

Body and Texture—35—Normal Range, 34.5–31.0

Gelatinous—A sticky, jelly-like character.
Mealy—A character of curd which resembles corn meal in the mouth.
Pasty—A soft, weak curd which resembles a flour-water paste.
Rubbery—An elastic, resilient curd.
Too firm—A curd which is hard and does not break easily in the mouth.
Weak—Curd which is soft and lacks body.

Color and Appearance

15—Normal Range, 14.5–11.0

Free whey—The presence of a thin, watery fluid.
Lacks cream—A dry curd lacking sufficient cream.
Lack uniformity—Lack of uniform-sized particles.
Matted—The agglomeration of two or more particles of curd.
Overcreamed—The presence of too much free cream.
Shattered curd—Curd which has been broken, owing to improper handling or packaging, and which may exhibit curd dust.
Slimy—A slippery film on the curd surface, due to chemical or microbial agents.
Surface discoloration—A noncharacteristic color on the surface, due to the growth of microorganisms.
Translucent—A lack of opaqueness, caused by chemical or microbial agents.
Unnatural color—Curd which does not present a creamy-white color.

Package—5—Normal Range, 5–4

Broken—A broken, torn, or damaged carton.
Container not full—A carton not containing the declared amount of cheese, or not full within ½-in. from the cover seat.
Insanitary closure—In which the closure is inset in the carton and not over the lip of the carton.
Leaky—A nonwaterproof carton, or one which leaks whey or cream. A soggy carton.
Soiled—A carton or closure presenting an unattractive exterior appearance, because of the presence of cheese, cream, or foreign matter.

REPORT OF REPRESENTATIVES OF THE AMERICAN DAIRY SCIENCE ASSOCIATION ON THE DAIRYWIDE COORDINATING COMMITTEE FOR NUTRITION RESEARCH[1]

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The Dairywide Coordinating Committee on Nutrition Research is composed of representatives of 15 associations, representing both dairy products manufacturers and producers' groups.

[1]R. G. Hansen usually served as Chairman, but he was in Europe at the time this report was prepared; therefore, it was submitted by J. D. Ingle and K. G. Weckel.

The Chairmanship of the Committee is rotated annually, alphabetically, among the participating organizations. Since January, 1957, the Committee has served under the chairmanship of H. F. Judkins, Sec.-Treas. of the American Dairy Science Association.

The objectives of the Dairywide Coordinating Committee are to provide assistance toward

better coordination of nutrition research in the dairy industry, especially toward greater use by the industry of established facts in nutrition. Specifically, they are:

1. Assembling and evaluating from available sources, consumer and professional attitudes toward dairy foods
2. Reviewing scientific data to provide factual background for public information about dairy foods
3. Suggesting additional needed nutrition research to encompass all phases of human nutrition as affected by dairy foods
4. Presenting to the dairy industry authentic information about dairy foods, and formulating recommendations for use in public relations, advertising, and educational work
5. Making available to dairy groups an annual summary of dairy industry–sponsored nutrition research projects in progress.

The operating headquarters of the Committee are in the offices of the National Dairy Council, which has furnished space and considerable staff and materials assistance.

The Committee operates most effectively through a number of subcommittees. The Committee meets twice yearly, in January and June, and the subcommittees more frequently. The subcommittees consist of *(a)* a research survey subcommittee; *(b)* an editorial subcommittee, to study the recommendations of the research survey subcommittee, to determine subject matter for articles for industry.

In 1953, a Subcommittee on Attitudes poll submitted a report on consumer attitudes, which has served as a basis of much of the Committee's attention. The principal points in the poll on attitudes were: *(a)* fat metabolism (reducing diets); *(b)* diseases; *(c)* effects of heating on milk; *(d)* calcium assimilation; *(e)* dental caries; *(f)* malnutrition, and *(g)* cholesterol. Three other phases of the poll were classified as: nutrition education, produce promotion, and field of economics.

The Research Survey Subcommittee in 1954 reported the following as subjects on which good information could be obtained, which could be used by the dairy industry for promotion: *(a)* Dairy foods in weight-reduction diets; *(b)* selling dairy foods on their protein value (R. G. Hansen, agreed to prepare three articles for use by industry, and the editorial subcommittee drew plans for effective merchandising of the articles in industry); *(c)* dairy foods for dietary fat; *(d)* dairy foods for malnutrition; *(e)* dairy foods in dental health, for calcium, and in utilization of calcium.

It presented a report of a Bibliography of Scientific Reports of Dairy Industry–Sponsored Nutrition Research.

In 1954, a report was made by the Research Survey Subcommittee on suggestions for a nutrition research program for the dairy industry. This report, rather detailed and lengthy, covered gaps in the information on milk and human nutrition, butterfat and fat-soluble factors, and whole milk and water-soluble nutrients.

In 1955, the Research Survey Subcommittee issued its revised report on "Recommendations for Nutrition Investigations of Primary Importance to the Dairy Industry." This was submitted to the Editor of the *Journal of Dairy Science*, who recommended certain revisions. Consideration was given to these suggestions by committee action. In the fall of 1955, Dr. Hansen issued a paper "Milk Protein—Invaluable Source of 'Body Building Blocks'," for release through the Committee. The Committee released also "Thirty-three Dairy Industry–Sponsored Nutrition Research Projects."

At the June 28, 1956 meeting, consideration was given to further recommendations for nutrition research, including human requirements of less well-known but well-studied essential nutrients, butterfat in production and processing, comparison of lysine-enriched bread and bread with nonfat milk solids, relative digestibility of butterfat and other fats, galactose metabolism and galactocemia, and infant feeding with reference to amino acid composition.

A second article by Dr. Zoe Anderson, "The Role of Dairy Foods in Weight Reduction and Control," was released through the Committee to the press for industry use.

In June, 1956, a special subcommittee was appointed to make recommendations to guide industry in using nutrition information in promotional programs and for establishing a recommended policy for ethical advertising in promoting dairy products. There appear to have been numerous instances of exaggerated or unsupported claims by the dairy industry for dairy products as nutritive foods. This subcommittee developed a proposed policy for consideration by the general committee at the June, 1957 meeting.

In July, 1956, the Research Subcommittee participated in a survey to help acquire information for bringing up to date, and more completely, the Agriculture Handbook No. 8, "Composition of Foods—Raw, Processed, Prepared," published by the U. S. Department of Agriculture and scheduled for revision.

The Research Subcommittee gave considerable thought to current nutrition and health problems affecting the dairy industry, including: *(1)* Implication of fat as a causative factor in heart disease, *(2)* alleged presence in milk of a virus acting as a causative factor in heart disease and cancer, *(3)* alleged presence of cancer-causing materials in dairy products resulting from carcinogens in plastic-coated milk containers and carcinogens in plastic coatings of containers for butter and cheese, and yellow coal-tar dyes, *(4)* implications of antibiotics in milk, and *(5)* utilization of milk calcium.

Members of the Subcommittee have participated in conferences at Loyola University, and in Chicago, on atherosclerosis and fats in the

diet. This subject is currently occupying a considerable portion of the efforts of the Research Subcommittee and of the Editorial Subcommittee on ways and means of providing the dairy industry with information to use in answering the public's questions. A meeting of the Coordinating Committee was scheduled for June of 1957.

The representatives of the American Dairy Science Association are members of the subcommittees of the Committee. The work of the committees, being entirely voluntary, is seemingly slow; the problems are ponderous, but apparently are best met through such means. Continued participation on the Committee by A.D.S.A. is recommended by its present representatives, all of whom have participated in at least six meetings during the year.

The résumé of the period prior to the past year has been presented solely to provide the Executive Board of A.D.S.A. with a background for current activities.

REPORT OF MEETING OF GOVERNING BOARD OF AMERICAN INSTITUTE OF BIOLOGICAL SCIENCE

H. D. EATON[1]

Connecticut Agricultural Experiment Station, Storrs[2]

The meeting of the Governing Board of the American Institute of Biological Science was held in the United Nations Room of the Student Union at the University of Connecticut, on August 25, 1956, and was called to order by President Glass.

Executive Secretary's report. After a few introductory remarks by President Glass, the Executive Secretary, Dr. H. T. Cox, gave his report. As an example of the possible service to member and affiliate societies, he indicated the role of A.I.B.S. in obtaining funds from the National Institutes of Health for the preparation of a 15-year Cumulative Index of the *Journal of Parasitology*. He further emphasized that A.I.B.S. wants to know the needs of its members and affiliate organizations so that it may do a better job. After Dr. Cox's report, methods of communicating the report to the membership, as well as the general purposes of A.I.B.S., were discussed.

1956 Activities of A.I.B.S. President Glass briefly indicated the many activities A.I.B.S. had undertaken during the past year. For example, he cited the aid of A.I.B.S. in providing travel for 40 U.S. scientists to the International Conference on Genetics held in Japan. A.I.B.S. also selected the persons who attended.

Authorized new activities. 1. *General Conference of Editors.* Based on recommendations of a preliminary conference of a small number of editors of biological journals, a proposal prepared by A.I.B.S. was submitted to the National Science Foundation (N.S.F.) for consideration, in May of 1956. This proposal indicated a conference of 50 editors in all fields of biology, except those medically oriented, with expenses for travel and subsistence to be paid by A.I.B.S. with funds provided by N.S.F. This conference was approved by N.S.F. and was scheduled for early in 1957.[3]

2. *Conference on Aging as a Biological Problem.* This conference was currently being evaluated by N.S.F.

3. *Visiting Lecturers' Programs.* A grant of funds from N.S.F. and the Atomic Energy Commission (A.E.C.) has provided A.I.B.S. with sufficient funds to provide 70 weeks of lecturing. The plan, in brief, is to provide a list of lecturers who are not only respected for their scientific status but who can also present biology in a favorable manner ("sell biology"). These lecturers will receive travel allowances and a modest honorarium, with the host institution providing lodging and meals. This program is intended for small liberal arts colleges and nearby institutions, e.g., high schools, biology clubs, etc. The duration of the lecturer's residence at the institution would not be less than three days and not more than seven. This program is on a year-to-year basis, its renewal dependent upon results. To date, biologists have viewed it with considerable favor.

After presenting these new activities, the selection of editors, conferees, and lecturers for these programs was discussed. This was based on the necessity of limiting the number for each program. It appeared to be the consensus, but was not unanimous, that some announcement of these programs should be made, for example in *Science*, for the purpose of allowing all those interested to apply. However, final selection will rest with the Committee charged with the duty of selection and in no way should limit the committee's selection only to those who applied. After further discussion

[1] Representative of the American Dairy Science Association, an affiliate of the American Institute of Biological Science.

[2] Published according to the policy stated, *J. Dairy Sci.*, **40**: 1369. 1957.

[3] This conference for biological editors was held in New Orleans, Louisiana, April 2–4, 1957. Problems considered were those relating to the economy and management of scientific journals, the selection and preparation of material for publication, the cooperation of journal editors with indexing and abstracting services, and the need for new publication media. *E. O. Herreid.*

as to whether or not this procedure should be put in the form of a motion, it was indicated that these were thoughts of the majority of the Governing Board and should be considered by the Executime Director in carrying out such programs.

Proposed activities. 1. *Quarterly Review of Biology.* Since the present editors of this Journal felt that they could no longer operate under the conditions imposed by Williams and Wilkins, the publishers, and the Waverly Press, printers (minimum need was one full-time editorial assistant, which could not be met by the publishers), it was sggested that the A.I.B.S. consider taking over the responsibilities of publication. The Williams and Wilkins Co. offered to sell publishers' rights to A.I.B.S. for $10, with the agreement that the Waverly Press print the Journal, with costs comparable to other printing, for a period of five years. The rights for publication or reprint of earlier issues (approximately 1925-1951) had been given to the Walter-Johnson concern of New York; this did not include extra back copies or reprint rights from 1951 on.

After lengthy discussion, it was moved and passed that A.I.B.S. accept Williams and Wilkins' offer, effective 1957, and that the A.I.B.S. Governing Board delegate authority to the A.I.B.S. Editorial Board, subject to the approval of the A.I.B.S. Executive Board, to appoint editors for five-year terms, beginning 1958.

2. *Translation of Russian Periodicals.* Since N.S.F. has supported translation into English of Russian chemical, mathematical, and physical publications, Director Cox explored the possibility of their support for biological publications. After checking the legal aspects of such activities, it was deemed desirable to have the work done on a contract basis by the Consultants Bureau of New York. After consideration of the pros and cons by the Board Members, it was moved and passed that the A.I.B.S. seek funds from N.S.F. for translating three Russian journals (Microbiology, Plant Physiology, and the Biological and Botanical Sections of the Proceedings of the Academy of Sciences of the U.S.S.R.) on a one-year trial basis. Dr. Cox indicated costs of $15 per page, which included translating, printing, and binding of approximately 1,000 copies. A.I.B.S. would then make these copies available to libraries or individuals.

3. *Otker Activities.* Group insurance, as presented in the Executive Director's report, was discussed. Suggestions as to a Biological Professional Calendar were received and discussed.

A representative from the Office of Naval Research (O.N.R.), a guest of the Board, talked about the great need for intercommunication between biologists. He suggested that the Board consider ways and means of increasing the exchange of written communication, as well as personnel. A motion was passed to set up an ad hoc committee to study this general problem and present definite suggestions to the International Exchange of Biological Information and Personnel.

The possible cooperation of A.I.B.S. with the Federation of American Societies for Experimental Biology, in undertaking a joint public relations effort to keep the public informed of biological findings, was outlined by President Glass. He stated that a committee had just been appointed from A.I.B.S. and was to begin immediately to explore the possibility of a joint effort by the two biological groups, A.I.B.S. and the Federation of American Societies for Experimental Biology. Representatives from N.S.F. and O.N.R. emphasized the lack of publicity in relation to biological activity, and the rather concerted efforts by the chemical, medical, and physical scientific groups. Some further discussion suggested that unless the public was informed, this would adversely affect federal, state, and other appropriations for biology in the future.

Relationship of A.I.B.S. to member and affiliated societies. Considerable discussion pertaining to this general problem was undertaken by the Board. It was this observer's feeling that A.I.B.S.'s objective was to become a representative for all biologists, by serving them in a manner somewhat similar to the American Chemical Society's method of serving chemists. Some of the Board felt that most biologists, belonging to member and affiliate societies, did not have an understanding of what A.I.B.S. was currently doing, or hoped to do in the future. It was requested that Executive Director Cox prepare material explaining A.I.B.S. activities, and distribute same to member and affiliate societies and others which might possibly be interested in A.I.B.S.'s activities.

Dr. Taylor, a guest representing the American Association for the Advancement of Seience (A.A.A.S.), pointed out the anticipated membership changes. It was moved and passed by the Board that A.I.B.S. become an affiliate, instead of an associate, member of A.A.A.S.

Site of 1959 meetings. Because of the scheduling of the International Botanical Conference the last two weeks of August, 1959, in Canada, some difficulty in choosing a site and time for the 1959 meeting of A.I.B.S. was anticipated. The Board suggested meeting in the southwestern part of the United States or on the East Coast prior to or after the International Conference. No need for formal action was indicated by President Glass, since the Executive Director desired only general viewpoints, to serve as a guide for future action.

Nominations. Nominations were made and approved as follows:

President: W. O. Fenn
Vice-President: P. J. Kramer
Secretary-Treasurer: F. P. Cullinan
Executive Committee Member: C. P. Swanson

With the exception of the Secretary-Treasurer, who was to take office immediately, the officers would begin serving January 1, 1957.

OFFICERS, COMMITTEES, AND REPRESENTATIVES OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

OFFICERS

President.....D. V. JOSEPHSON, The Pennsylvania State Univ., University Park
Vice-President.....K. L. TURK, Cornell Univ., Ithaca, N. Y.
Secretary-Treasurer.....H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y.
Journal Editor-in-Chief.....E. O. HERREID, Univ. of Illinois, Urbana

DIRECTORS

C. F. HUFFMAN, Michigan State Univ., East Lansing
(Immediate Past President)

E. L. FOUTS, Univ. of Florida, Gainesville (1958)
I. W. RUPEL, Texas A & M College, College Station (1958)
G. M. WERNER, Univ. of Wisconsin, Madison (1959)
E. H. PARFITT, Evaporated Milk Association, 228 N. LaSalle St., Chicago (1959)
E. L. JACK, Univ. of California, Davis (1960)
H. A. HERMAN, National Association of Artificial Breeders, Columbia, Mo. (1960)

STANDING COMMITTEES

Policy

W. V. PRICE, *Chairman*, Univ. of Wisconsin, Madison, Wis. (1960)

I. A. GOULD, The Ohio State Univ., Columbus (1960)
F. J. ARNOLD, Iowa State College, Ames (1959)
J. H. ERB, The Borden Company, Columbus, Ohio (1959)
R. E. HODGSON, USDA, Beltsville, Md. (1959)
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

Auditing

W. H. LIST, *Chairman*, Association of Ice Cream Manufacturers,
405 Lexington Ave., New York 17, N. Y.

RANDALL WHITAKER, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
C. B. BENDER, The Sperry Corporation, 30 Rockefeller Plaza, New York 20, N. Y.

Ballot Tabulating

G. A. BOWLING, *Chairman*, Strathglass Farm,
Port Chester, N. Y.

ALEX C. HERRO, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
H. W. HOWARD, The Borden Company, 350 Madison Ave., New York, N. Y.

Historian

G. M. TROUT, Michigan State Univ., East Lansing

Journal Management

W. M. ROBERTS, *Chairman*, North Carolina State College, Raleigh

D. H. JACOBSEN, 20 N. Wacker Dr., Chicago, Ill.
J. T. REID, Cornell Univ., Ithaca, N. Y.
E. O. HERREID, Univ. of Illinois, Urbana (ex officio)
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

Membership

J. B. FRYE, JR., *Chairman*, Louisiana State Univ., Baton Rouge

W. M. SNYDER, *Associate Chairman*, Student Affiliate Membership, Michigan State Univ., East Lansing

W. S. ARBUCKLE, *Chairman*, Eastern Division, Univ. of Maryland, College Park
L. R. DOWD, *Associate Chairman*, Eastern Division, Univ. of Connecticut, Storrs
H. E. CALBERT, *Chairman*, Central Division, Univ. of Wisconsin, Madison
E. L. THOMAS, *Associate Chairman*, Central Division, Univ. of Minnesota, St. Paul
L. H. RICH, *Chairman*, Rocky Mountain Region, Utah State Univ., Logan
GEORGE STODDARD, *Associate Chairman*, Rocky Mountain Region, Utah State Univ., Logan
H. A. BENDIXEN, *Chairman*, Western Region, State College of Washington, Pullman
F. B. WOLBERG, *Associate Chairman*, Western Division, Oregon State College, Corvallis
P. M. REAVES, *Chairman*, Southern Division, Virginia Polytechnic Institute, Blacksburg
J. E. JOHNSTON, *Associate Chairman*, Southern Division, Louisiana State Univ., Baton Rouge
R. R. RIEL, Science Service, Ottawa, Canada
J. E. MOXLEY, McDonald College of McGill Univ., Quebec, Canada
D. L. GIBSON, Univ. of Saskatchewan, Saskatoon, Canada

Necrology

T. B. HARRISON, *Chairman*, Univ. of Tennessee, Knoxville

K. M. AUTREY, Alabama Polytechnic Institute, Auburn
P. L. KELLY, Univ. of Nebraska, Lincoln
D. J. HANKINSON, Univ. of Massachusetts, Amherst
N. L. VANDEMARK, Univ. of Illinois, Urbana
G. H. WILSTER, Oregon State College, Corvallis
J. J. WILLINGHAM, Texas Technological College, Lubbock

Public Relations

F. J. GREINER, *Chairman*, Ohio Dairy Products Association, 5 E. Long St., Columbus 15, Ohio (1959)

J. HOFFMAN ERB, The Borden Company, 165 N. Washington Ave., Columbus, O. (1958)
R. H. RUMLER, The Holstein-Friesian Association, Brattleboro, Vt. (1958)
P. E. NEWMAN, Beacon Milling Company, Cayuga, N. Y. (1959)
R. B. MAXCY, The Diversey Corporation, Chicago, Ill. (1960)
W. L. DUNKLEY, Univ. of California, Davis (1960)

Nominating

F. J. ARNOLD, *Chairman*, Iowa State College, Ames

C. F. HUFFMAN, Michigan State Univ., East Lansing
O. F. GARRETT, M & R Dietetic Laboratories, 625 Cleveland Ave., Columbus, O.
L. L. RUSOFF, Louisiana State Univ., Baton Rouge
A. J. MORRIS, Utah State Agricultural College, Logan

Program

N. P. RALSTON, *Chairman*, Michigan State Univ., East Lansing

F. J. BABEL, Purdue Univ., West Lafayette, Ind.
S. P. MARSHALL, Univ. of Florida, Gainesville
L. R. FRYMAN, Univ. of Illinois, Urbana
J. W. POU, North Carolina State College, Raleigh
C. F. HUFFMAN, Michigan State Univ., East Lansing

Resolutions

D. M. SEATH, *Chairman*, Univ. of Kentucky, Lexington

R. E. LEIGHTON, Texas A & M College, College Station
C. H. PARSONS, Univ. of Massachusetts, Amherst
J. C. FLAKE, Evaporated Milk Association, Chicago, Ill.
E. B. OBERG, Carnation Company, Los Angeles, Calif.

Student Affiliate

W. W. SNYDER, *Chairman*, Michigan State Univ., East Lansing (1960)

J. E. JOHNSTON, Louisiana State Univ., Baton Rouge (1958)
L. R. DOWD, Univ. of Connecticut, Storrs (1958)
W. L. SLATTER, The Ohio State Univ., Columbus (1959)
P. M. REAVES, Virginia Polytechnic Institute, Blacksburg (1959)
F. G. WARREN, North Carolina State College, Raleigh (1960)

Public Health

L. A. BLACK, *Chairman*, U. S. Public Health Service,
4676 Columbia Parkway, Cincinnati 26, Ohio

A. C. DAHLBERG, Cornell Univ., Ithaca, N. Y.
W. D. POUNDEN, Ohio Agricultural Experiment Station, Wooster
W. A. KRIENKE, Univ. of Florida, Gainesville
E. W. BIRD, Iowa State College, Ames
C. F. HUFFMAN, Michigan State Univ., East Lansing
E. B. KELLOGG, Milk Industry Foundation, Washington 6, D. C.
B. M. ZAKARIASEN, Land O'Lakes Creameries, Inc., Minneapolis 13, Minn.
(Additional industry and public health members to be added)

Divisions

D. J. HANKINSON, *Chairman*, Univ. of Massachusetts, Amherst

K. M. AUTREY, Alabama Polytechnic Institute, Auburn
L. H. RICH, Utah State Agricultural College, Logan
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y. (ex officio)

AWARD COMMITTEES

American Feed Manufacturers Award

J. C. SHAW, *Chairman*, Univ. of Maryland, College Park (1959)

S. R. SKAGGS, New Mexico College of Agriculture, State College (1958)
G. H. WISE, North Carolina State College, Raleigh (1958)
N. N. ALLEN, Univ. of Wisconsin, Madison (1959)
W. A. COWAN, Univ. of Connecticut, Storrs (1960)
J. F. SYKES, USDA, Bureau of Dairy Industry, Beltsville, Md. (1960)

Borden Award—Manufacturing

F. E. NELSON, *Chairman*, Iowa State College, Ames (1958)

S. T. COULTER, Univ. of Minnesota, St. Paul (1958)
E. O. HERREID, Univ. of Illinois, Urbana (1959)
G. H. HARTMAN, White House Milk Company, Manitowoc, Wis. (1959)
STUART PATTON, The Pennsylvania State Univ., University Park (1960)
BURDET HEINEMANN, Producers Creamery Company, Springfield, Mo. (1960)

Borden Award—Production

G. W. SALISBURY, *Chairman*, Univ. of Illinois, Urbana (1958)

C. W. DUNCAN, Michigan State Univ., East Lansing (1958)
H. E. BECHTEL, Larro Research Farm, Indianola, Iowa (1959)
H. D. EATON, Univ. of Connecticut, Storrs (1959)
I. R. JONES, Oregon State College, Corvallis (1960)
J. F. CAVANAUGH, American Jersey Cattle Club, Columbus, Ohio (1960)

DeLaval Extension Award

L. H. STINNETT, *Chairman*, Oklahoma State Univ., Stillwater (1958)

GEORGE HYATT, JR., North Carolina State College, Raleigh (1958)
F. J. ARNOLD, Iowa State College, Ames (1959)
E. C. SCHEIDENHELM, Univ. of Kentucky, Lexington (1959)
C. W. REAVES, Univ. of Florida, Gainesville (1960)
H. R. SEARLES, Univ. of Minnesota, St. Paul (1960)

Honors

L. A. MOORE, *Chairman*, USDA, Agricultural Research Service, Beltsville, Md.

I. A. GOULD, Ohio State Univ., Columbus
C. F. HUFFMAN, Michigan State Univ., East Lansing

The Dairy Science Teaching Award

N. P. TARASSUK, *Chairman*, Univ. of California, Davis (1958)

C. E. WYLIE, Univ. of Tennessee, Knoxville (1958)
J. T. REID, Cornell Univ., Ithaca (1959)
F. H. HERZER, Mississippi State College, State College (1959)
G. M. TROUT, Michigan State Univ., East Lansing (1960)
C. L. NORTON, Oklahoma State Univ., Stillwater (1960)

SPECIAL COMMITTEES

Education

W. L. SLATTER, *Chairman*, Ohio State Univ., Columbus

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C. C. OLSON, North Dakota State Agricultural College, Fargo
J. T. LAZAR, JR., Clemson Agricultural College, Clemson, S. C.
E. L. JACK, Univ. of California, Davis (ex officio)

Personnel Manual Preparation

H. B. HENDERSON, *Chairman*, Univ. of Georgia, Athens

I. A. GOULD, The Ohio State Univ., Columbus
H. J. WILLIAMS, Carnation Company, Los Angeles, Calif.
H. F. JUDKINS, 32 Ridgeway Circle, White Plains, N. Y.
F. H. GRAY, Southern Dairies, Inc., Charlotte, N. C.
F. C. EWBANK, Michigan Milk Producers' Assn., 355 East Second Street, Imlay City, Mich.

Association Representatives

American Association for the Advancement of Science: J. W. THOMAS, Bureau of Dairy Industry, USDA, Beltsville, Md.

Committee on Inter-Society Cooperation: L. A. MOORE, Dairy Husbandry Research Branch, USDA, Beltsville, Md.

Dairy Remembrance Fund: D. V. JOSEPHSON, *Chairman*, The Pennsylvania State Univ., University Park; J. C. SHAW, Univ. of Maryland, College Park; A. C. DAHLBERG, Cornell Univ., Ithaca, N. Y.

Dairy-wide Coordinating Committee on Nutrition Research: H. F. JUDKINS, *Chairman*, 32 Ridgeway Circle, White Plains, N. Y.; J. D. INGLE, 9825 S. Peoria Street, Chicago, Ill.; G. C. NORTH, Beatrice Foods Company, 1526 S. State Street, Chicago, Ill.

National Research Council: W. E. KRAUSS, Ohio Agricultural Experiment Station, Wooster

Ralston-Purina Research Fellowship Committee: J. B. FRYE, JR., Louisiana State Univ., Baton Rouge

The Sub-Committee on Education and Training of the Dairy Industry Committee Representative: H. B. HENDERSON, Univ. of Georgia, Athens

American Institute of Biological Sciences Representative: D. L. HILL, Purdue Univ., West Lafayette, Ind.

3-A Sanitary Standards Committee: J. R. BRUNNER, Michigan State Univ., East Lansing

U. S. Livestock Sanitary Association: W. D. KNOX, *Hoard's Dairyman*, Fort Atkinson, Wis.

National Association of Artificial Breeders: J. O. ALMQUIST, The Pennsylvania State Univ., University Park

OFFICERS AND COMMITTEES OF THE MANUFACTURING SECTION

SECTION OFFICERS

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B. H. WEBB, *Vice-Chairman*, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
STUART PATTON, *Secretary*, The Pennsylvania State Univ., University Park

SECTION COMMITTEES

Program

F. J. BABEL, *Chairman*, Purdue Univ., West Lafayette, Ind.
B. H. WEBB, National Dairy Research Laboratories, Oakdale, L. I., N. Y.
STUART PATTON, The Pennsylvania State Univ., University Park

Butter

T. R. FREEMAN, *Chairman*, Univ. of Kentucky, Lexington
E. W. BIRD, Iowa State College, Ames
H. J. BUYENS, 1436 North Lawler, Chicago, Ill.
CLAUDE HARPER, JR., Beatrice Foods Co., 1526 S. State St., Chicago 5, Ill.
S. R. HOOVER, Eastern Regional Research Laboratory, Philadelphia, Pa.
CHRIS JENSEN, North Dakota Agricultural College, Fargo

Nomenclature and Methodology of Milk Proteins

B. L. LARSON, *Chairman*, Univ. of Illinois, Urbana
J. R. BRUNNER, Michigan State Univ., East Lansing
ROBERT JENNESS, Univ. of Minnesota, St. Paul
N. P. TARASSUK, Univ. of California, Davis
C. H. WHITNAH, Kansas State College, Manhattan
R. M. WHITNEY, Univ. of Illinois, Urbana
C. A. ZITTLE, Eastern Utilization Research Branch, 600 E. Mermaid Lane, Philadelphia 18, Pa.

Dairy Products Judging

G. M. TROUT, *Chairman*, Michigan State Univ., East Lansing
C. J. BABCOCK, USDA, Washington, D. C.

L. R. Dowd, Univ. of Connecticut, Storrs
P. A. Downs, Univ. of Nebraska, Lincoln
E. L. Thomas, Univ. of Minnesota, St. Paul
F. C. Warren, North Carolina State College, Raleigh
J. J. Willingham, Texas Technological College, Lubbock

Sub-Committee to Develop a Score-Card for Nonfat Dry Milk

E. L. Thomas, *Chairman*, Univ. of Minnesota, St. Paul
F. E. Fenton, USDA, Washington, D. C.
Ray Powers, The Borden Company, New York, N. Y.
R. P. Choi, American Dry Milk Institute, 221 N. LaSalle St., Chicago, Ill.
W. K. Stone, Virginia Polytechnic Institute, Blacksburg
N. P. Tarassuk, Univ. of California, Davis
B. M. Zakariasen, Land O'Lakes Creameries, Inc., Minneapolis 13, Minn.

Curd Tension of Milk

L. K. Crowe, *Chairman*, Univ. of Nebraska, Lincoln
U. S. Ashworth, State College of Washington, Pullman
L. W. Aurand, North Carolina State College, Raleigh
J. H. Hetrick, Dean Milk Co., 1126 Kilborn Ave., Rockford, Ill.
H. A. Rishoi, Cherry Burrell Corp., 426 W. Randolph St., Chicago, Ill.

Resolutions

J. C. Flake, *Chairman*, 325 Callan St., Evanston, Ill.
V. H. Nielsen, Iowa State College, Ames
C. E. Parmelee, Purdue Univ., West Lafayette, Ind.

Nominations

C. L. Hankinson, *Chairman*, Ohio State Univ., Columbus
H. E. Calbert, Univ. of Wisconsin, Madison
O. F. Garrett, M & R Dietetic Laboratories, 625 Cleveland Ave., Columbus, O.

OFFICERS AND COMMITTEES OF THE PRODUCTION SECTION

SECTION OFFICERS

S. P. Marshall, *Chairman*, Univ. of Florida, Gainesville
N. L. Jacobson, *Vice-Chairman*, Iowa State College, Ames
G. W. Trimberger, *Secretary*, Cornell Univ., Ithaca, N. Y.

SECTION COMMITTEES

Dairy Cattle Judging

H. E. Kaeser, *Chairman*, The Ohio State Univ., Columbus (1958)
W. R. Murley, North Carolina State College, Raleigh (1959)
G. B. Marion, Kansas State College, Manhattan (1960)

Guidance in Conduct Production Section Session

R. E. Erb, *Chairman*, State College of Washington, Pullman (1958)
N. P. Ralston, Michigan State Univ., East Lansing (1959)
S. W. Mead, Univ. of California, Davis (1960)

Program

S. P. MARSHALL, *Chairman*, Univ. of Florida, Gainesville
N. L. JACOBSON, Iowa State College, Ames
G. W. TRIMBERGER, Cornell Univ., Ithaca, N. Y.

Resolutions

R. E. LEIGHTON, *Chairman*, Texas A & M College, College Station
A. E. DRACY, South Dakota State College, College Station
R. E. MATHER, Rutgers Univ., New Brunswick, N. J.
D. J. HILL, Purdue Univ., West Lafayette, Ind.

JOINT COMMITTEES OF THE PRODUCTION AND EXTENSION SECTIONS

EXTENSION	PRODUCTION

Breed Relations

(Chairman in Production each year)

RALPH WAYNE, Univ. of Minnesota, St. Paul (1958)	K. A. HUSTON, *Chairman*, Kansas State College, Manhattan (1958)
C. H. BOYNTON, Univ. of New Hampshire, Durham (1959)	C. F. FOREMAN, Iowa State College, Ames (1959)
G. E. GORDON, Univ. of California, Berkeley (1960)	H. W. THOELE, Pennsylvania State Univ., University Park (1960)

Dairy Cattle Breeding

(Chairman in Extension each year)

S. N. GAUNT, *Chairman*, Univ. of Massachusetts, Amherst (1958)	L. D. MCGILLIARD, Univ. of Michigan, East Lansing (1958)
H. W. CARTER, Cornell Univ., Ithaca, N. Y. (1959)	R. C. LABEN, Univ. of California, Davis (1959)
C. D. MCGREW, Ohio State Univ., Columbus (1960)	L. O. GILMORE, Ohio State Univ., Columbus (1960)

Type

(Chairman alternates. From Production in 1958)

J. G. CASH, Univ. of Illinois, Urbana (1958)	J. W. POU, *Chairman*, North Carolina State College, Raleigh (1958)
RAY ALBRECTSEN, Cornell Univ., Ithaca, N. Y. (1959)	F. W. ATKESON, Kansas State College, Manhattan (1959)
CLYDE CHAPPELL, Univ. of Tennessee, Knoxville (1960)	C. L. BRANTON, Louisiana State Univ., Baton Rouge (1960)

Dairy Cattle Health

(Chairman alternates. From Production in 1958)

C. G. BRADT, Cornell Univ., Ithaca, N. Y. (1958)	J. C. SHAW, *Chairman*, Univ. of Maryland, College Park (1959)
E. T. ITSCHNER, Univ. of Missouri, Columbia (1959)	H. J. HILL, Colorado A & M College, Fort Collins (1958)
F. H. EWALT, Oregon State College, Corvallis (1960)	W. A. KING, Clemson Agricultural College, Clemson, S. C. (1960)

OFFICERS AND COMMITTEES OF THE EXTENSION SECTION
1957–1958

SECTION OFFICERS

L. R. FRYMAN, *Chairman*, Univ. of Illinois, Urbana
L. A. JOHNSON, *Vice-Chairman*, Michigan State Univ., East Lansing
C. W. NIBLER, *Secretary*, Univ. of Nebraska, Lincoln

SECTION COMMITTEES

Dairy Records

D. E. VOELKER, *Chairman*, Iowa State College, Ames (1959)
J. W. CROWLEY, Univ. of Wisconsin, Madison (1958)
H. C. GILMORE, Pennsylvania State Univ., University Park (1958)
MARVIN SENGER, North Carolina State College, Raleigh (1960)
E. R. BONEWITZ, Kansas State College, Manhattan (1960)

Resolutions

CLARENCE PARSONS, *Chairman*, Massachusetts State Univ., Amherst (1959)
C. W. NIBLER, Univ. of Nebraska, Lincoln (1958)
W. R. VAN SANT, Univ. of Arizona, Tucson (1960)

Teaching Methods

J. D. GEORGE, *Chairman*, North Carolina State College, Raleigh (1960)
L. H. STINNETT, Oklahoma State Univ., Stillwater (1959)
M. F. ELLMORE, Virginia Polytechnic Institute, Blacksburg (1959)
W. R. HESSELTINE, Connecticut State Univ., Storrs (1958)

4-H Club

W. N. PATTERSON, *Chairman*, Virginia Polytechnic Institute, Blacksburg (1958)
W. P. ANDERSON, Pennsylvania State Univ., University Park (1959)
A. M. MEEKMAN, A & M College of Texas, College Station (1960)
C. C. OLSON, North Dakota State College, Fargo (1960)

ABSTRACTS OF PAPERS

PRESENTED AT THE WESTERN DIVISION MEETING

PULLMAN, WASHINGTON, JULY 14-17, 1957

A Method for Selecting Drying Conditions for the Determination of the Total Solids in Milk. U. S. ASHWORTH, State College of Washington, Pullman.

A standard milk may be prepared accurately from spray-dried powder of known composition. This milk can then be used to verify the drying rate of an oven which is being used for total solids determinations of fresh or concentrated milks. A curve may be prepared by plotting the calculated per cent total solids remaining after any drying time in the oven against time. The point where this curve crosses the known value for total solids in the standard milk solution gives the optimum drying time for the conditions being employed. The standard milk has special value in checking any of the recently developed rapid drying methods for total solids in milk.

Factors Affecting the Dye Binding of Milk Proteins. U. S. ASHWORTH AND R. SEALS, State College of Washington, Pullman.

A rapid and simple procedure for the estimation of protein in milk is based on the reaction between the acid dye, Orange G, and the basic groups of the milk proteins. The only reagent needed is one containing 0.1% of the dye and 2.1% of citric acid. Fresh milk or powder containing 20-50 mg. of protein was shaken with 25 ml. of the dye and allowed to stand for about 1 hr. The protein-dye complex was then removed either by filtering or by centrifuging. The optical density of the clear filtrate was read at 420 mμ. A special flow-through light absorption cell is described. This cuvette has a 1-mm. length light-path which made further dilution of the dye unnecessary.

The error due to absorption of dye by the filter paper is small and compensated for by filtering the standard. In the temperature range between 46 and 130° F. no significant effect of temperature on dye-binding could be observed. Whey proteins gave higher binding values (mg. dye per gram of protein) than did casein. Alpha-casein bound more dye than beta-casein. The dialyzable nitrogen fraction bound no dye.

Colorimetric Determination of Lactose in Milk Using the Phenol-Sulfuric Acid Reaction. D. R. ERICKSON AND G. A. RICHARDSON, Oregon State College, Corvallis.

A rapid and simple colorimetric method for the determination of lactose in milk is described which gives results in agreement with the official polarimetric method. It consists of mixing 10 ml. of milk diluted with 100 ml. of water, with 7 ml. of 5% dialyzed iron, bringing to 250 ml. volume with distilled water, and filtering. The resulting clear filtrate is 1 to 100, and 2 ml. of this was transferred to a 19 by 105 mm. cuvette. One ml. of a 5% phenol solution was added to the cuvette, followed by 5 ml. of concentrated H_2SO_4 C. P. The developed color was read in a spectrophotometer at 490 mμ. The color was stable and followed the Beer-Lampert law. Data showed that the method gave results in statistical agreement with the official polarimetric method, when the technique, especially the rate of addition of the sulfuric acid, is standardized.

Plastics for Hydrometry. N. S. GOLDING, Professor Emeritus, State College of Washington, Pullman.

The objective of this study has been to develop special types of plastic lactometers, to determine the density of whole milk, skimmilk, and whey.

The conventional glass lactometers have two disadvantages:

1. They are fragile and the more accurate they are made the more fragile they become; a larger sample of milk must then be used.
2. The coefficient of thermal expansion of glass is many times less than water, making accurate temperature control essential.

Several plastics lower in density than most wheys do not have these disadvantages. These plastics have been studied for their suitability for hydrometers and most of them have one or more desirable properties. However, polymethylstyrene (CYMAC-400) thus far has all the physical properties necessary for making an accurate hydrometer.

When CYMAC-400 is mixed cold and then mixed hot with very fine barium sulphate and a small amount of insoluble color, pressure-injection molded discs or spheres of any required density have been made by varying the percentage of barium sulphate. The advantage of this plastic is that it has the same thermal expansion as water at 20 to 25° C. Annealed discs of CYMAC-400 maintained their exact density in milk when changed and washed weekly for 4 mo.

Panels as a Means of Determining Consumer Acceptance of Dairy Products. R. R. HEDRICK, J. L. BRENCE, AND J. A. NELSON, Dairy Industry Department, Montana State College, Bozeman.

Panels have been used to a limited extent in determining consumer acceptance of dairy products, and more extensively to determine consumer preferences of other foods. In se-

lecting the panel, experience, availability, age, sex, health, taste and smell sensitivity, reliability of judgment, and other psychological factors have been taken into consideration. The size of the panel has been governed by availability and cost; the larger the panel, the more reliable the results, but a panel with a small number of members with ability to taste is considered more conducive to accuracy than a large one with low taste abilities. Importance has been placed on the management of the panel, condition and manner in which the samples are presented to the panel members, and the condition of the judging room. For more accurate results, panels have been organized so that the data can be analyzed statistically.

Paper Electrophoresis of Casein. L. M. Libbey and U. S. Ashworth, State College of Washington, Pullman.

A study of the paper electrophoresis of casein was made with buffers of about pH 12. A KOH-K_2HPO_4 buffer of pH 12.3 and ionic strength of 0.19 clearly resolves casein into three fractions, in the Spinco apparatus run at 20-40 milliamperes for 30 hr., preferably at about 4° C. The level of sample application was critical; 0.01—0.02 ml. of an approximately 10% protein whole casein sol is recommended. To achieve the highest degree of resolution, the sample was placed as near as possible to the cathode by cutting a special slot in the Durrum type cell. After electrophoresis, the paper strips were dyed with bromphenol blue. Without correcting for tailing errors, the relative distribution of the casein fractions, based on areas beneath scanning curves, was approximately 50% alpha- and kappa-, 45% beta-, and 5% gamma-casein.

Effect of Ingredients of Chocolate Drink on Consumer Acceptance. H. S. Willard, W. R. Thomas, and W. D. Schutz, University of Wyoming, Laramie.

On the basis of chi-square values, a panel of 40 or more students showed a definite preference for 1% fat over no fat, 3% fat over 1% fat, 11% solids-not-fat over 9% solids-not-fat, vanillin flavoring over pure vanilla flavoring, 7% sugar over 3% sugar, and 1% over 1.5% Dutch Process cocoa. The students were about evenly divided on preference of high- and low-viscosity chocolate drinks. The panel used both the hedonic scale and the rank-order method of expressing preference of three samples at each taste session. The rank-order scale method proved to be more sensitive than the hedonic scale method, but the latter brought out the degree of dislikes and likes. The basic mix in each test consisted of 93.98% milk testing 2% butterfat, 1.24% Dutch Process cocoa, 4.75% beet sugar, and 0.03% stabilier. Improvement in score and a decrease in the percentage dislikes resulted by adding 0.7% vanillin and 7% beet sugar, and decreasing cocoa to 1%.

Determination of the Fat Content of Cottage Cheese with Modified Babcock Methods. E. A. Zottola and R. W. Stein, Oregon State College, Corvallis.

Four series of tests were run, to find a satisfactory method for testing creamed cottage cheese for fat. In three of the series, tests were made on pints of creamed cottage cheese obtained from local grocery stores; the fourth series was run on cottage cheese creamed in the laboratory. Five methods were studied: *(1)* ether extraction, *(2)* D.P.S. detergent, *(3)* butyl alcohol, *(4)* Minnesota modification, and *(5)* Pennsylvania modification. In this study, the ether extraction method was assumed to give correct results and was the standard. The data showed that of the four methods, only the butyl alcohol method compared favorably with the ether extraction method. On laboratory-perpared samples, the butyl alcohol test showed lower fat readings than the ether extraction method, except in products containing more than 5% fat, in which the reverse was true.

Effects of Adding Seminal Plasma to Concentrated Sperm Samples at 37° C. J. L. Albright, M. H. Ehlers, and R. E. Erb, State College of Washington, Pullman.

Seminal plasma is used as a vehicle for spermatozoa and supplies energy for their metabolism. A study was conducted to determine whether washed sperm-citrate samples, devoid of extracellular nutrients and showing marked declines in motility, could utilize plasma nutrients.

By centrifugation, sperm from 23 samples were removed and twice-washed with sodium citrate buffer. Restoration to original plasma volume was with sodium citrate and is referred to as semen-equivalent. The 0.2 ml. semen-equivalent plus 0.8 ml. citrate samples was placed in a 37° C. water bath. Progressive motility estimates were recorded initially and at hourly intervals for 4 hr. before and after the addition of 0.1 and 0.2 ml. of seminal plasma.

Results were as follows:

	Hours postincubation				
	Initial	1	2	3	4
Before adding 0.1 plasma	24	6	1	0	0
After adding 0.1 plasma	—	26	20	13	3
After adding 0.2 plasma	—	40	38	20	23
Excess, adding 0.8 ml. plasma	65	56	48	39	33

Concentrated sperm-citrate samples that showed little or no progressive motility and appeared to be dead, could be restimulated by adding nutrients in seminal plasma.

Alternate Five-Day Injections of Oxytocin as a Means of Maintaining Maximum Milk Production. D. V. ARMSTRONG AND G. M. WARD, Department of Dairy Industry, Colorado State University, Fort Collins.

This study was initiated to determine whether intramuscular injections of oxytocin would maintain production in hard-milking cows which showed a low degree of persistency. The effect upon milking rate was also investigated. Five hard-milking cows were selected: two Brown Swiss, two Holsteins, and one Guernsey, and injected intramuscularly with either 30 or 40 U.S.P. units of oxytocin immediately before milking. The injections were continued for five days and followed by a five-day control period, after which this procedure was repeated. The study covered from 100 to 130 days.

The average increase in daily milk production for the period of injection over the control period for the five cows was 1.6, 0.7, 1.6, 1.2, and 4.3 lb. The average milking time for all of the cows was reduced from 8.4 min. during the control period to 6.5 min. during the periods of injection. The injections had no effect upon the regularity of heat periods.

Performance of Lactating Dairy Cows and Carrying Capacity of Pastures as Influenced by Supplemental Feeding and Seasonal Pasture Growth. C. W. ARAVE, C. H. MICKELSON, G. E. STODDARD, AND G. Q. BATEMAN, Utah State University, Logan.

During the summer, pasture forage from an improved seeding was clipped and fed to four groups of Holsteins. They were fed individually all the clipped pasturage they would eat and, in addition, Group 1 received 1 lb. of grain for each 5 lb. of milk; Group 2 received 1 lb. of grain for each 10 lb. of milk; Group 3 received no grain and hay; and Group 4 received 10 lb. of hay daily, of which 9.1 was consumed.

Groups 1 to 4 consumed 1,010, 420, 0, and 0 lb. of grain, respectively, during 122 days. Group 4 consumed 1,106 lb. of hay. Cows in Groups 1, 2, 3, and 4 produced 4,274, 3,689, 3,881, and 4,144 lb., respectively, of 4% FCM which, when corrected for differences in production during a ten-day preliminary period, amounted to 4,274, 4,465, 3,661, and 4,323 lb. of 4% FCM. There were no measurable differences in body weight, and all groups increased in weight. Yields of dry matter for silage or hay were comparable.

Effect of Continuous Feeding of Slaked Lime and Vitamin A to Calves on a Diet of Reconstituted Whey, Grain, and Hay to 60 Days of Age. D. C. BROWN, H. S. WILLARD, AND J. W. HAMILTON, University of Wyoming, Laramie.

Two series of experiments with 28 and 35 Holstein calves, respectively, in each series, were conducted to determine if the incidence of intestinal irritation and dry, scaly skin could be reduced when slaked lime was fed to prevent scour. When the liquid part of the ration was reduced from 10 to 6 lb. daily, from 30 to 60 days of age, there was no indication that slaked lime acted as an intestinal irritant. Scaly skin was noted in all whey-fed calves, but to a lesser extent in calves which started to eat grain at an early age. The scaly skin condition disappeared soon after the calves began eating from ¾-lb. to 1 lb. of grain daily. Sixteen calves fed a capsule containing 12,000 units of vitamin A in 4 ml. of pure cottonseed oil each day between 20 and 60 days of age, had sleeker coats than the controls, but the group receiving the vitamin A had scours more often and their growth rate was less than the control group. The authors believe that the increase in rate of scouring of the vitamin A–fed calves was due to the cottonseed oil carrier. Statistical analysis of the data indicated that sex of calf was not related to treatment.

Relationship of Seminal Fructose and Free Amino Acids Concentration to Sexual Maturation of the Bull. F. X. GASSNER AND M. L. HOPWOOD, Colorado State University, Fort Collins.

In studies previously reported, it was shown that seminal free amino acids rise in the adolescent bull and reach the adult pattern at about 20 mo., then remain plateaued to at least 12 yr. of age.

It was also shown that seminal fructose was present at a near-mature level at 11 mo. of age, indicating that the Leydig Cell system begins to function before spermatogenesis occurs.

In order to establish at what age seminal fructose appears and reaches the adult level, seminal fluid was collected by electroejaculation from 26 bull calves, 7 to 10 mo. old. Fructose and free seminal amino acids were determined by methods previously reported.

Seminal fructose concentration steadily increased from 27 mg/100 ml. at 7 mo. to 65 mg/100 ml. at 9 mo. of age. At 11 mo., it was 370 mg/100 ml. and at 12 mo., it reached 580 mg/100 ml., which is considered the mature level. Therefore, maturation of the Leydig Cell system, when measured by fructose production, occurred at least 3 mo. before the mature pattern of seminal amino acids and spermatogenesis began to become established.

Free Amino Acid Concentration in Bovine Testes and Accessory Sex Organs. M. L. HOPWOOD AND F. X. GASSNER, Colorado State University, Fort Collins.

The free seminal amino acid pattern of maturing bulls became apparent by the 15th

mo. of age, reaching a plateau at 20 mo. Seminal fluid of the immature bull lacks glutamic acid, which appears in direct proportions to sperm production. While amino acids disappear after castration or vasectomy, all except glutamic acid reappear following testosterone treatment. Since it seems that glutamic acid is primarily a testicular product and is also related to spermatogenesis, it became of interest to trace its origin.

Testes, epididymides, ampullae, and seminal vesicles from ten normal bulls were obtained and, after expressing fluid from all but the testes, were frozen until used. Ethanol extracts were chromatographed on paper to determine the amino acids.

Testes showed the highest concentration of glutamic acid. Fluid from epididymides was most concentrated. Fluids and tissues of the ampullae and seminal vesicles, however, were richer in alanine. This indicates that the organs producing and storing up to and through maturation elaborate the high concentration of glutamic acid found in the ejaculate. It also is indicated that glutamic acid fulfills a function on which normal development of spermatozoa depends.

Influence of Altering the Amount of Plasma on Semen Metabolism. N. S. KUSHWAHA, M. H. EHLERS, AND R. E. ERB, State College of Washington, Pullman.

Semen glycolysis was studied by varying the amount of seminal plasma. Each of 28 samples from three bulls was divided into three portions: I, whole semen; II, semen with volume reduced to one-half by removal of much of the plasma; and III, semen with volume doubled by added plasma. The citrate-buffered portions were incubated at 37° C., with fructose and lactic acid being determined at 0, ½, and 1 hr. After ½ hr. incubation, fructose utilization based on sperm numbers was about ⅓ higher in I and III than in II. At 1 hr., utilization was highest in III. Lactic acid formation was greatest in III for both incubation intervals.

This experiment emphasizes the twofold problem in selecting an incubation time. An incubation of ½ hr. at 37° C. will underestimate glycolytic rate for samples having a combination of low fructose and high sperm concentration. Samples with high fructose and low sperm concentration, however, may have excessive errors in measurement with an incubation of ½ or 1 hr.

Intermittent Colostrum Feeding and Its Effect upon Blood Plasma, Viatmin A, Carotene, Calcium, and Phosphorus of Dairy Calves. E. A. KEYES, E. J. PEACE, AND J. L. BRENCE, Dairy Industry Department, Montana Agricultural Experiment Station, Bozeman.

Thirty-six Holstein calves were divided at birth and fed in three groups for 80 days. Group I was fed colostrum and whole milk intermittently. Group II was fed colostrum the first four days and Holstein whole milk from different cows intermittently, and Group III was fed colostrum the first four days and Holstein milk continuously from one cow. Colostrum was substituted for milk of equal weight, with no scouring effect due to the colostrum. The calves were weighed and total digestible nutrients were computed on the feed eaten. Blood analyses were made for vitamin A, carotene, calcium, and phosphorus. Samples were taken on the fourth, 16th, and 20th days. There was no visible difference between the calves in Groups I and II or Groups II and III. There was no significant difference between any of the three groups of calves when total digestible nutrients consumed were compared with gain in weight. Blood plasma analyses indicated no significant difference between groups. Growth rates were normal. Results indicate that equal weights of colostrum may be substituted for milk in feeding young dairy calves, without causing any detrimental effects or changing the blood plasma analyses from normal calves.

A Comparison of Conventional and Self-Feeding Dairy Cows for the Honolulu Milk Market. K. K. OTAGAKI AND K. MORITA, University of Hawaii, Honolulu.

Good-quality forages generally supply the most economical source of nutrients, including vitamins, minerals, and proteins. However, the extended use of pasture and cultivated soilage crops in Hawaii is limited, because of the tremendous urbanization of the existing farm areas and the resulting scarcity of desirable agricultural areas for economical forage production.

It might be feasible to increase the concentrates and to feed no more green roughage than is necessary to prevent animals from going off feed and to provide a proper mixture for efficient rumen activity.

Feeding trials with minimum roughage feeding, accompanied by self-feeding of concentrates, showed profitable returns over feed costs, when compared with the conventional twice-a-day feeding. Better milkers have produced at higher levels and have maintained milk production over an extended period. The self-fed lots also showed improvement in general appearance and body weight gains at the termination of the experiment.

Nutritive Value of Molasses-Bagasse Pith Mixtures when Fed to Dairy Heifers. P. A. PUTNAM AND T. H. BLOSSER, State College of Washington, Pullman.

Six yearling Jersey heifers, 4 mo. in gestation, were used to determine the apparent digestibility and TDN contents of 2:1 and 4:1 molasses-bagasse pith mixtures, when fed

with chopped alfalfa hay. After a ten-day preliminary period and a seven-day collection period, during which the digestibility of alfalfa was determined, the heifers were divided into two groups and fed 6 lb. daily of the molasses-bagasse mixtures. Digestibility was determined by difference, using the chromic oxide indicator method and grab samples of feces collected three times daily. The average TDN content of the hay on an as-fed basis was 43.0 (49.7 on a 100% dry basis). The 2:1 molasses-bagasse mixture contained 45.1% TDN (56.2 on a 100% dry basis) and the 4:1 mixture 62.8% TDN (80.3 on a 100% dry basis).

Corn Steep Liquor as a Preservative for Grass Silage. J. R. SCHUBERT, J. V. BATEMAN, I. R. JONES, AND P. H. WESWIG, Oregon State College, Corvallis.

Three one-ton silos were filled with second-cutting alfalfa and grass, one untreated, one with 70 lb/T molasses and one with 100 lb/T corn steep liquor.[1] The untreated silage (pH 5.08) underwent extensive butyric acid deterioration. This was considerably improved by adding molasses (pH 4.62) or corn steep liquor (pH 4.70). The respective nonvolatile/volatile acid ratios were 0.49, 0.92, and 0.72. The silages were fed to pairs of dairy heifers for five-day, total collection digestion trials. Digestion coefficients of 60.1, 60.5, and 66.9 for the dry matter contents were obtained for the untreated (26.3% D.M.), molasses (28.1% D.M.), and corn steep liquor (25.1% D.M.) silages, respectively. Similarly, crude protein contents of 11.6, 11.6, and 14.0%, dry basis, showed 62.6, 60.2, and 74.6 digestion coefficients. T.D.N. values of 58.9, 57.9, and 63.8 were calculated, the latter reflecting improved digestibility of each of the proximate principles of corn steep liquor silage. Corn steep liquor appears to be a potential preservative for grass sligae, based on enhanced feeding value, as well as a nutrient source for microorganisms.

[1] Generously provided by the A. E. Staley Manufacturing Company, Decatur, Illinois. Solids, 50%; pH, 3.9; $N \times 6.25$, 21.9%; lactic acid 9.8% (wet basis).

Comparative Value of Alfalfa Hay and Alfalfa Silage as Winter Roughage for Dairy Cows. G. E. STODDARD, C. H. MICKELSEN, AND C. Q. BATEMAN, Utah State University, Logan.

During 1956, third-crop alfalfa from 16.72 acres was harvested, half as silage and half as hay. Alfalfa for silage was chopped directly from the stump and ensiled in an upright concrete stave silo, half without preservative and half with sodium metabisulfite, at the rate of 8 lb. per ton of green weight. Both hay and silage were of very good quality. Forage fed was 2,504 lb. of air dry matter for the hay and 2,581 lb. for the silage.

Twelve cows were paired and assigned to one of three groups: Group 1, hay and corn silage; Group 2, hay and alfalfa silage, and Group 3, alfalfa silage and corn silage. Results indicated that dry matter consumption from alfalfa silage was not as great as from either alfalfa hay or corn silage. Milk production was not maintained as well when alfalfa silage replaced either hay or corn silage in the ration. No differences in body weight were observed.

All-Roughage vs. Roughage and Concentrate Feeding for Holstein Steers. R. O. RUPP, G. M. WARD, AND M. H. HAZALEUS, Colorado State University, Fort Collins.

As a part of a larger project, this study was undertaken to determine the effect of adding concentrates to the ration of Holstein steers on *(1)* the returns above feed cost, *(2)* the rate of gain, *(3)* the finish at 1,000 lb., *(4)* the length of time required to reach 1,000 lb., and *(5)* the percentage of wholesale cuts.

Green chopped alfalfa, alfalfa hay, and corn silage were fed and the grain consisted of one-half corn and one-half dried beet pulp. The steers were 1 yr. of age when the feeding trial began.

The grain-fed group was slaughtered 100 days later and the all-roughage group 156 days later. The grain-fed and all-roghage fed steers, respectively, weighed 1,004 and 1,031 lb. at slaughter, gained 2.7 and 1.8 lb. per day, dressed 53.1 and 53.3%, and the returns above feed cost were $14 and $10. The percentage of wholesale cuts and of separable bone, fat, and lean was almost the same for both groups. All of the animals in both groups graded U. S. Standard.

Carotene and Vitamin A Content of Bovine Liver Before and After Parturition. P. H. WESWIG, I. R. JONES, R. G. SPROWLS, AND J. F. BONE, Oregon Agricultural Experiment Station, Corvallis.

The carotene and vitamin A content of livers of 15 normal cows from the station herd was determined from biopsy specimens. Average carotene and vitamin A values were 12.0 γ/g and 129.0 γ/g liver, respectively, 4 wk. before parturition, and 12.6 γ/g and 120.0 γ/g liver 4 wk. postpartum. With carotene intakes ranging from 130 to 390 γ per kilo body weight, twelve livers averaged 1.5 γ/g and 13.6 γ/g liver carotene and vitamin A, respectively, 4 wk. before parturition, and 1.3 γ/g and 12.8 γ/g liver 4 wk. postpartum. Blood and milk fat samples were taken at the time of biopsy. Also, in selected instances, carotene and vitamin A were determined on colostrum.

No significant changes in prepartum and

postpartum liver contents were noted in either group. Comparisons over periods longer than 2 mo. may reflect seasonal variation in normal animals. Twenty-four parturitions occurred when liver stores were maximum (July-December). It appears that liver stores of carotene and vitamin A do not change significantly during the period 4 wk. before or after parturition.

Effect of Stage of Lactation on the Solid Components of Milk from Individual Holstein-Friesian Cows. W. S. NICHOLSON, JR., W. R. THOMAS, H. S. WILLARD, AND D. C. BROWN, University of Wyoming, Laramie.

This paper describes the variations observed in 10 mo. lactation of 18 cows for 3 yr. Samples were collected monthly from individual cows during two consecutive milkings. Total solids were determined by the Dietert method, fat by the Babcock method, and solids-not-fat by difference. Analysis of variance of the monthly yield of milk, solids-not-fat, and total solids showed significant differences between cows at the 1% level. The differences in monthly yields during lactation were found to be the result of both linear and quadratic regression. Butterfat yields were due solely to regression. Butterfat percentage did not conform statistically either to linear or to quadratic regression. Solids-not-fat and total solids percentage data paralleled closely those for butterfat percentage. However, the level of significance for quadratic regression and for combined linear and quadratic regression was only 5%. The data indicate that it is difficult to fit a smoothed curve from percentages of milk constituents obtained over a lactation period.

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ABSTRACTS OF LITERATURE

W. O. Nelson, Abstract Editor

BOOK REVIEWS

1. Methods of Biochemical Analysis. Vol. 5. Ed., DAVID GLICK. Interscience Publishers, New York. 502 pages. $9.50. 1957.

The fifth volume of Methods of Biochemical Analysis continues to maintain the high level of quality characteristic of previous volumes in this series. All the contributors are recognized authorities in their field and have covered their subjects well. In keeping with the objective of the series as a review series in methods and techniques, the articles discuss both the background and the basis of the various subjects, as well as give detailed procedures for actual determinations. Thus, the series is of use as a reference work for the biochemist, as well as being of value to the scientist of allied disciplines in need of specific biochemical methods and techniques to use in specific instances. Examination of the book suggested that not as many subjects or methods have been covered in this volume as in previous volumes. This was confirmed by the useful cumulative author-and-subject index for the first five volumes located in the back of the book. What the volume lacks in number of subjects covered, however, is amply made up for by the completeness of the subjects covered.

Methods and subjects covered in this volume are: Assay Methods for Cholinesterases, by Klas-Bertil Angustinsson; Biological Standards in Biochemical Analysis, by J. H. Humphrey, D. A. Long, and W. L. M. Perry; α-Keto Acid Determinations, by William J. P. Neish; Microdetermination of Cobalt in Biological Materials, by Bernard E. Saltzman and Robert G. Keenan; Activation Analysis and Its Application in Biochemistry, by B. A. Loveridge and A. A. Smales; Contamination of Trace Element Analysis and Its Control, by Ralph E. Thiers; Chemical Determination of Estrogens in Human Urine, by W. S. Bauld and R. M. Greenway, and The Infrared Analysis of Vitamins, Hormones, and Coenzymes, by Harris Rosenkrantz, followed by the Author and Subject Indexes for Volume 5, and the Cumulative Indexes. B. L. Larson

2. Tracer Applications for the Study of Organic Reactions. JOHN G. BURR, JR. Interscience Publishers, New York. 291 pages. 1957. $7.50.

In publishing this book, the author feels that he is helping to fill a void brought about by the rapid increase in the use of isotopic tracers to examine the nature and results of organic reactions. He feels that the use of isotopic tracers in organic chemistry has not adequately been made available to the newcomer in this area, as evidenced by the small number of comprehensive reviews and texts available. By providing a survey of tracer applications in organic chemistry and some generalizations on the principles of isotopic tracer use, it is hoped that the reader will be able to judge intelligently the merits of the methods and, in so doing, this will stimulate the greater use of isotopes among organic chemists.

The Table of Contents lists 13 chapters. These are: General Considerations for the Use of Isotopic Tracer Methods, A Discussion of Isotopic Exchange Reactions, Proton Transfer in Nonaromatic System, Nucleophilic Displacements in Nonaromatic Substances, Exchange Reactions in Aromatic Systems, Free Radical Processes, Carbonium Ion Processes, Molecular Rearrangements with Specific Mechanisms, Reactions of the Carbon-Oxygen Bond, The Oxidation of Aliphatic Molecules, Polymerization Reactions Miscellany, and The Structure of Molecules and Stable Intermediates.

Each chapter is well-subdivided on the basis of specific actions or reactions. For example, the chapter on Molecular Rearrangements with Specific Mechanisms includes ten categories such as the Willgerodt Reaction, The Benzidine Rearrangement, and the Curtius Rearrangement, followed by suitable references. The chapter on Exchange Reactions in Aromatic Systems also includes ten categories such as Employment of the Hydrogen Isotope Effect, Nature of the Aromatic Electrophilic Substitution Intermediate, also followed by suitable references.

The book is well-referenced and the several appendices include general texts and references as well as newer references accumulated after the body of the text was written. These are quite useful and bring the book up to date. This book should be of use chiefly to the theoretical organic chemist interested in the study of reaction mechanisms. The implications of the reaction mechanisms discussed also make it of use to the biochemist concerned with the possible mechanism of specific biological reactions. B. L. Larson

3. **Drying Farm Crops.** CARL W. HALL. Agricultural Consulting Associates, Inc., Reynoldsburg, Ohio. 1957. 336 pages. $7.

This book meets a demand created by the rapidly increasing farm crop drying industry. It is written to serve both as a text for class work and a reference for anyone interested in this field. Much research data is accumulated from widely located sources and presented in tables and curves in an accessible manner. Each subject lists an adequately complete bibliography for further investigation by the reader.

The subject matter is divided into ten chapters covering various phases of the general topic. Following each chapter is a list of problems suitable for use in class work. An appendix includes a great deal of general information on crop drying.

The opening chapter covers the importance of drying farm crops, including the prevention of field losses and the retention of quality both in the harvest and in storage. The economic advantages of drying farm crops also are considered.

Basic principles underlying drying are covered at some length. These include equilibrium moisture content between air and grain, moisture changes, heat transfer, and allied subjects. Drying farm crops with both unheated and heated air is covered at some length.

Practical methods of drying various grains are explained in some detail and numerous installations that might be used on the farm are described. Farm hay drying, with various methods of accomplishing it, is covered rather completely.

Commercial equipment that is available for farm drying is listed and, in some instances, prices are shown. The text is illustrated with many sketches of drying apparatus.

J. H. Ramser

4. **Manual for Milk Plant Operators.** Milk Industry Foundation, Washington, D. C. 2nd ed. 1957. 670 pages. $8.

Forty-three persons, representing the dairy industry, equipment manufacturers, and educational institutions, have contributed to this revised manual. It contains 39 chapters, which are divided into seven parts as follows: I. Plant layouts and maintenance. II. Utilities; water and its purification; power; refrigeration and waste disposal. III. Milk plant equipment; selection; metals; bulk tank transportation; pasteurizers; automatic recorders, and pallet systems. IV. Fundamentals; chemistry and physics of milk products; bacteriology and defects in milk and cream. V. Quality control; judging and testing; processing and pasteurizatoin; by-products and specialties; losses and accounting. VI. Housekeeping; cleaning and maintenance of equipment; insect and rodent control. VII. Personnel; accident prevention; selecting, training, and supervising personnel.

This manual is written clearly and simply and it will be useful for the practical man in the market milk plant; but other sources of information will be needed for the modern specialized plant, where all the common milk products are processed and manufactured. It is not likely to inspire or stimulate the well-trained young men in the dairy industry, because advanced concepts are conspicuously absent. E. O. Herreid

5. **Proceedings of National Conference on Bulk Handling of Milk.** Departments of Dairy and Agricultural Engineering, Michigan State University, East Lansing.

The objectives of this conference were to bring together outstanding authorities and to have them present the most recent information on the bulk handling of milk. The ideas and information contained in the conference papers cover all phases of bulk tank handling, including standard terms and definitions, reasons for bulk handling, every-day vs. every-other-day pick-up and effects on milk quality, installing tanks and determining their accuracy, pipeline handling of milk, owner vs. contract hauling, sanitizing tanks and regulating haulers, fieldman's responsibility, and economic considerations.

Bulk tanks have proved superior to anything yet invented to transport milk from the farm to the processing plant. This compilation of papers contains useful and practical information that should be available to all plant personnel engaged in bulk milk handling or who are about to adopt this latest system.

Copies of these papers may be obtained from Professor C. W. Hall, Department of Agricultural Engineering, Michigan State University. E. O. Herreid

BUTTER

6. **Continuous buttermaking.** ANON. Milk Prod. J., **48**, 9:14. 1957.

The Co-operative Dairy Association in Kansas produces two million pounds of butter annually, using a continuous process. Only farm-separated sour cream is used. The cream is neutralized to a predetermined acidity which results in the most efficient separation. The 35% cream is preheated to 135-145° F. and separated into an oil testing 80% butterfat.

The oil is pumped to a phase reverser which breaks the fat from the serum, and the mixture of oil and serum is passed through a serum separator which yields a 96-98% butter oil.

The purified oil is processed through an HTST pasteurizer and then vacuumized. This oil, now 99.6% pure, is plate-cooled to 130° F. and discharged into composition vats where proper butter mix is prepared. The ready-mix then is pumped to an initial chiller, where it receives some working. The mix is cooled by sweet water to a temperature of just under 60° F., then discharged into a large chiller with two chambers and two dashers. Cooling is accomplished with ammonia, lowering the temperature of the mix to 40-42° F. From the chillers, the mix goes to a crystallizer, where the product is subjected to a controlled working process which controls crystallization. The butter may be printed directly or held in bulk. J. J. Janzen

7. Paper butter crocks boost sales. ANON. Milk Prod. J., **48**, 9: 17. 1957.

The introduction of the one- and two-pound, round, paper butter crock resulted in increased sales. The paper crocks are polyethylene-lined. Colors are gray, yellow, and white. The rim of the crock is bright yellow. Sales messages appear on side of container.
J. J. Janzen

8. Butter dispenser. C. W. DUNNET, U. S. Patent 2,810,195. 3 claims. Oct. 22, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 4:652. 1957.

Pats or individual portions of butter are cut from a large block and delivered on a plate ready for serving, which is positioned below the dispenser. R. Whitaker

9. Butter dispenser. L. R. PUERNER. U. S. Patent 2,809,428. 9 claims. Oct. 15, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 3:449. 1957.

A stick of butter is inserted in a magazine which feeds it to a cutter for dispensing individual pats or portions. R. Whitaker

10. Butter and cheese holder and slicer. E. J. MORLASCHI. U. S. Patent 2,814,113. 1 claim. Nov. 26, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 4:659. 1957.

Prints of butter or cheese are placed in this rectangular-shaped holder and moved toward one end for slicing at an adjustable rate into individual-size portions. R. Whitaker

11. Combination butter and cheese cutter, server, and storer. M. ACKERMAN. U. S. Patent 2,813,336. 3 claims. Nov. 19, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 3:453. 1957.

A portion of butter or cheese as, for example, a quarter-pound print, is inserted in this device. A plate at one end pushes the print toward the other end where individual portions can be sliced off as desired.
R. Whitaker

CHEESE

12. High-viscosity cottage cheese dressing. L. J. MANUS, Dept. of Dairy Sci., State Coll. of Wash., Pullman. Milk Prod. J., **48**, 10:56. 1957.

The author discusses the problems involved in the preparation of a high-viscosity cottage cheese dressing without the use of stabilizers. The dressing contained 12.5% butterfat, and a total solids content of not less than 20%. With this fat content and the use of proper homogenization temperatures and pressures, extreme fat-clumping and a high viscosity may be produced in the dressing. This increase in viscosity makes the dressing cling to the curd, coating each particle.

Three tables are presented which show the relationship of homogenizing temperatures and pressures on the finished product.

Some factors influencing the development of the desired viscosity include rehomogenization, previously homogenized products, homogenizing temperature, cooling and rewarming of the product, agitation while cooling, and storing for 24 hr. at 45-40° F.

Processing directions also are presented.
J. J. Janzen

13. Processing of Cheddar cheese. H. C. JACKSON, JR. (assignor to Armour and Co.) U. S. Patent 2,813,028. 4 claims. Nov. 12, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 2:365. 1957.

Green curd is extruded by means of a worm-type extruder into individual casings, where it ripens at an above-normal rate.
R. Whitaker

14. Cheese-making apparatus. A. E. NESSLER AND J. G. HARTMAN (assignors to National Dairy Products Corp.) U. S. Patent 2,814,114. 8 claims. Nov. 26, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 4:659. 1957.

A method is described of securing and releasing various devices used in cutting and agitating cheese curd in a round, vertical cheese vat. R. Whitaker

15. Cheese manufacture. A. M. LEDERER (assignor to Swift & Co.). U. S. Patent 2,816,036. 4 claims. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2:329. 1957.

Cheddar cheese, made in the usual manner, is quick-frozen for at least an hour prior to the usual aging step. R. Whitaker

16. Manufacture of cheese slices. R. D. TRELEASE (assignor to Swift & Co.). U. S. Patent 2,815,573. 1 claim. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2:213. 1957.

Process cheese is molded into slices by feeding the hot cheese to a large chilled roll having depressions of the desired shape and size. An endless belt, operating at the same speed as the roll surface and in the same direction, and pressed against it by a series of chilled small rollers on the other side of the belt,

closes the mold. After about one-half revolution of the large roll, the belt leaves the roll, cane operated ejectors discharge the portions of chilled cheese from the depressions or molds, depositing them on the belt, to be conveyed to a packaging machine. R. Whitaker

CONDENSED AND DRIED MILKS; BY-PRODUCTS

17. Pliofilm-lined cheese box (cheese mold). C. Pauly (assignor to Swift & Co.). U. S. Patent 2,815,708. 2 claims. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2:248. 1957.

A combination cheese hoop and aging or shipping container, using a pliofilm liner.
R. Whitaker

18. Use of sucrose in the preservation of frozen condensed skimmilk. R. W. Bell, Eastern Util. Research and Development Div., USDA, Washington. Milk Prod. J., **48**, 9:18. 1957.

Skimmilks of 9 and 18% solids content, in the preparation of which the fluid skimmilk was held at 62.7° C. for 30 min., became oxidized within 1 mo. when stored at −12° and at −17° C. A heat treatment of 63.8° C. for 30 min. prevented oxidation from taking place in otherwise identical milks for a period of 3 mo. in the case of 1:1 milks and nearly 6 mo. in the 2:1 milks. Low solids skimmilk requires more heat than conventional pasteurization, for it to retain its flavor stability at −17 to −12° C. storage. High solids skimmilks, 3:1 and 4:1, have a high resistanee to oxidation at these temperatures. High concentration skimmilks receiving conventional pasteurization heat treatments retain flavor stability for 6 mo. or longer. However, body deterioration becomes more pronounced with the higher heat treated milks.

More sediment was noticeable in samples having received the higher heat treatment, regardless of the storage temperature or the addition of sucrose. Upon reconstituting, the samples containing sucrose redispersed more readily.

Storage temperatures of −17 and −12° C. were satisfactory, for body stability, at the higher solids levels only when 5 to 10% sucrose was added. A table is included giving combinations of heat treatment, quantity of sucrose added, and period of satisfactory preservation at various frozen storage temperatures for various concentrations. When sucrose was added after, rather than before, condensing, lower viscosities were obtained which facilitated handling before and after frozen storage. J. J. Janzen

19. Inert gas packaging. Anon. Milk Prod. J., **48**, 10:13. 1957.

Soluble dairy powders consisting of a blend of sweet cream, modified solids from skimmilk, lactose, and disodium phosphate are packed "under glass" in an inert gas atmosphere. The packaging material used is a lamination of pliofilm, aluminum foil, and pouch paper. This material enters the sealed-in packaging machine, is formed into pouches with heat-sealed edges, and is filled at speeds of up to 30, 1-lb. packages a minute.

Adjustment of the equipment can be made by the operator via rubber sleeves, with gloves on the end which permit the operator to reach into the filling section without permitting entry of air or escape of nitrogen.
J. J. Janzen

20. Process and composition for improving casein. R. B. Dean (assignor to the Borden Co.). U. S. Patent 2,810,655. 2 claims. Oct. 22, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 4:770. 1957.

A hard, horny plastic is formed from casein by dampening 100 parts casein and from one to ten parts by weight of an alkali metal polyphosphate with 3.40 parts of water. The dampened mass at 140-250° F. is passed between a pair of rollers spaced 0.03-0.25 in. apart. One roll operates at a peripheral speed of 1.125 to four times the other, to provide a shearing action. The rolling is continued until all graininess disappears, and is then cooled to form the plastic. R. Whitaker

21. Process for preparing a casein gel. M. L. Anson and M. Pader (assignors to Lever Brothers Co.). U. S. Patent 2,813,794. 5 claims. Nov. 19, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 3:571. 1957.

A chewy, fibrous-like casein, for use in food products simulating meat, is produced by preparing a casein solution at pH 6.8, heating to 70° C., adding a soluble calcium salt to give a calcium ion concentration of about 0.1 normal, then adjusting the pH to about 6.0 and heating to form a gel. R. Whitaker

DAIRY BACTERIOLOGY

22. Fermentation and equipment design. Ind. and Eng. Chem., **49**, 8:1215. 1957.

A symposium consisting of seven papers on the subject of fermentology is presented. These papers are of interest to the dairy technologist working in the area of whey utilization, since the fermentation of lactose has been an important method for converting this carbohydrate to other useful products. The subjects discussed in detail include, new process control applications in fermentation; automatic antifoam and nutrient feed control for bench scale fermentation; pH control in submerged pure culture fermentations; oxygen absorption in agitated tanks; citric acid production by submerged fermentation; characterization of agitation effects in shaken flasks, and oxygen absorption rates in shaken flasks.
B. H. Webb

23. An inquiry into the usefulness of the Standard Methods direct microscopic count. D. LEVOWITZ, N. J. Dairy Labs., New Brunswick. J. Milk and Food Technol., **20**, 288. 1957.

Direct microscopic counts do not give a numerical accuracy in raw milks because of *(a)* small number of cells present and uneven distribution; *(b)* solids deposition, and *(c)* personal interpretation of smear areas examined.

The author believes it is not a good practice to assume that Standard Methods direct microscopic count, or any modifications proposed to this date, will replace Standard Methods agar plate count in the examination of raw milk for pasteurization.

H. H. Weiser

DAIRY CHEMISTRY

24. The chemical composition and the cell count of milk. R. WAITE AND P. S. BLACKBURN, Hannah Dairy Research Inst., Kirkhill, Ayr, Scotland. J. Dairy Research, **24**, 328. 1957.

Approximately 3,000 samples of milk from 180 pairs of dams and their daughters were collected during a lactation. Bulk milk samples were taken at a rate of 16 each week for a year. The milk samples were analyzed for solids, solids-not-fat (difference), crude protein, casein, lactose, and fat. Lymphocytes, polymorph and epithelial cells were counted in breed smears.

Cell counts varied between 10,000 and five million per milliliter. There was considerable variation in chemical composition of milk with similar cell counts. As the cell count rose, the proportion of polymorph cells increased. As the cell count approached 0.5 million/ml, there was a fall in production and in solids-not-fat per cent of milk. The drop in solids-not-fat was due mainly to a decreased lactose, until counts of 1.0 million were approached, at which counts casein content also was affected. Fat content apparently was not affected. It was concluded that cows with counts of less than 100,000 cells/ml from full milkings would not be considered to be suffering from subclinical mastitis and that subclinical mastitis, as evidenced by cell count of milk, leads to a lowering of the solids-not-fat content.

J. D. Donker

25. Formamide as an extraction solvent in Karl Fischer method for determining moisture in lactose and maltose. E. A. MCCOMB, West. Reg. Research Lab., Albany, Calif. Analyt. Chem., **29**, 9:1375. 1957.

This note describes the use of formamide to replace methanol as the extraction solvent for determination of moisture in foods containing lactose or maltose, using the Karl Fischer method. Both water of crystallization and adsorbed water in these sugars can be determined. Good results were obtained on the monohydrates of lactose and maltose when the Fischer and oven methods were compared.

B. H. Webb

26. Determination of tyrosine and tryptophan in proteins. W. L. BENCZE AND K. SCHMID, Dept. of Med., Harvard Med. School, and Mass. Gen. Hospital, Boston. Analyt. Chem., **29**, 8:1193. 1957.

A spectrophotometric method for tyrosine and tryptophan determination, based on measuring absorbance in the range between 278 and 293 mμ, is described. Spectrophotometric determination of these amino acids in proteins is advantageous because it does not require hydrolysis, which often leads to partial decomposition, which is characteristic of other chemical methods.

B. H. Webb

27. Determination of soluble whey protein by direct nesslerization of Kjeldahl digests. W. K. STONE AND A. H. HOLMES, Va. Agr. Expt. Sta., Blacksburg. Food Research, **22**, 5: 501. 1957.

A method is presented for estimating soluble whey protein (as such, or in terms of nitrogen or in percentage of SNF) in heated milk products. An appropriate sample is extracted by saturated salt solution, the soluble proteins precipitated with $ZnSo_4$, digested with H_2SO_4, and nesslerized in the same tube. Optical density is measured in a spectrophotometer or other suitable instrument, the results obtained from a standard curve, and calculated to the desired basis.

F. J. Doan

DAIRY ENGINEERING

28. Treatment of cream. G. A. DUMMETT (assignor to A.P.V. Co., Ltd.). U. S. Patent 2,812,253. 2 claims. Nov. 5, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 1:146. 1957.

A cream deodorizer is described consisting of a series of closed chambers through which the cream and steam pass, countercurrently in some and, finally, co-currently. The pressure in the chambers decreases in the direction of flow, thus inducing movement of the cream from chamber to chamber.

R. Whitaker

29. Freezer mechanism. L. A. M. PHELAN. U. S. Patent 2,810,557. 9 claims. Oct. 22, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 4:747. 1957.

Construction details are given for a horizontal-type ice cream freezer. R. Whitaker

30. Horizontal spray drier. R. O. HENSZEY AND R. R. HENSZEY. U. S. Patent 2,815,071. 19 claims. Dec. 3, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 1:76. 1957.

Structural details are given for a horizontal type of spray drier. R. Whitaker

DAIRY PLANT MANAGEMENT AND ECONOMICS

31. Milk carton holder. M. E. KRUEGAR. U. S. Patent 2,810,503. 1 claim. Oct. 22, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 4: 732. 1957.

A device for holding a quart-size paper milk bottle with a handle, to facilitate pouring of the milk. R. Whitaker

32. Carrying device for containers. J. E. SOCKE (assignor to American Can Co.). U. S. Patent 2,809,861. 1 claim. Oct. 15, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 3:562. 1957.

A handle for carrying two Canco-type paper milk bottles side by side. R. Whitaker

33. Container liner. D. E. FORD AND L. P. FORD. U. S. Patent 2,815,887. 2 claims. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2: 292. 1957.

A milk can with a small hole in the bottom is lined with a disposable pliofilm, bag-like liner having in addition to the open top an outlet in the form of a plastic tube which is inserted through the hole. The arrangement is suitable for use in a dispenser, the liner collapsing as the product is withdrawn through the bottom outlet. R. Whitaker

FEEDS AND FEEDING

34. The effect of variations in nutrient intake upon the yield and composition of milk. I. Variations in the amount and type of concentrates fed in winter rations. A. W. A. BURT, N.I.R.D., Univ. Reading, England. J. Dairy Research, **24**, 283. 1957.

Five feeding trials were conducted in which the effects of various levels of concentrate feeding were tested. Groups of 18 Ayrshire, Dairy Shorthorn, or Holstein cattle were used. In the first experiment, a randomized block design with 4-wk. periods was used. In the remainder of the experiments, a changeover design was employed, using 3-wk. periods. As a result of additional grain feeding, approximately 0.7 to 1.0 of extra milk was obtained per pound of additional starch equivalent (S.E.). There was evidence that higher yielders responded more favorably than lower yielders and that the response from a second addition of 20% extra concentrate was only about half the response from the first 10% extra. It was necessary to add protein to balance the protein energy ratio to obtain response to extra feed. A substitution on a S.E. basis of limited quantities of hay for concentrates had no effect on milk production.

Solids-not-fat in the milk was affected in three of the five experiments by level of feeding. The animals with the lowest natural content of solids-not-fat were least affected by higher rate of feeding. J. D. Donker

35. The effect of variation in nutrient intake upon the yield and composition of milk. II. Factors affecting rate of eating roughages and responses to an increase in the amount of concentrate fed. A. W. A. BURT, N.I.R.D., Univ. of Reading, England. J. Dairy Research, **24**, 296. 1957.

Twenty-nine Ayrshire cattle were divided into a group of heifers and a group of older animals, to measure the rates at which individual cows consumed hay, kale, and concentrates. Measurements were conducted during two periods of 2 wk. time and were separated by an interval of 5 wk. There were significant differences between animals and days, and a significant cow × period interaction in rates of eating in both groups. The rate of eating kale decreased as the dry matter content of it increased, but as it increased in dry matter content, the consumption of kale dry matter increased in rate. There were positive associations between rates of consumption of the three feedstuffs. The live weight but not milk production was associated with rate of eating. Stage of lactation and climatic conditions did not seem to influence eating rate. It was determined that measurement for from four to five days was sufficient to give satisfactory measurements.

As additional concentrate was given, more milk was obtained and this was not dependent upon stage of lactation in this study. Cows with low solids-not-fat in their milk responded to the greatest degree to extra concentrate feeding. J. D. Donker

GENETICS AND BREEDING

36. Notched ears in New Zealand dairy cattle. M. A. MACDONALD, Ruakura An. Research Sta., Hamilton, New Zealand. J. Heredity, **48**, 5: 244. 1957.

Notched ear, which is thought to be the same trait as described by Yamane in Ayrshires, was studied in several herds of purebred and grade Ayrshire herds in the North Island of New Zealand. Two major classes of notched ears, i.e., severely and moderately notched, were distinguished and were concluded to be the resulting phenotypes of the homozygous and heterozygous pairing of alleles, respectively. The thickening of aural cartilage with this trait is discussed. Notched ears were found in cattle of both sexes. This aural abnormality is not discriminated against in the New Zealand show ring as in the United States as reported by Snyder, nor was it associated with type score. No relationship between notched ear and milk production (FCM) was found. L. O. Gilmore

HERD MANAGEMENT

37. The influence of the maximum temperature rise in bulk tanks on milk quality. P. J. VAN DEMARK AND P. R. MARCH. Dept. of Dairy Ind., Cornell Univ., Ithaca, New York. J. Milk and Food Technol., **20**, 11: 317. 1957.

The effect of lipase activity was studied when warm milk was added to cold milk. No rancidity was detectable at blend temperatures of 40, 50, and 60° F. However, it was noticeable at 70° F. and pronounced at 85° F.

The increment in acid degree values between 24 and 48 hr. is much less with every-other-day pick-up than with samples picked up daily.

It was observed that warming cold raw milk to 85° F. and recooling will stimulate lipase activity and cause rancidity. H. H. Weiser

38. The effect of milking practices upon the deterioration of milking machine inflations. G. M. Waid, V. R. Elsey, and J. A. Hurry, Colo. State Coll., Fort Collins. J. Milk and Food Technol., **20**, 11: 312. 1957.

Summer pasture or rations had little effect on rubber liners as compared to winter rations.

Storage of liners in 10% lye solution increased the time required to slow blistering. Pure fatty acids accelerated blistering.

Udder ointments and mastitis treatments used in this research showed no appreciable effects on the rubber parts of the milking machine. H. H. Weiser

39. The relationship between milking rate and milk yield. Oystein Sandvik, Inst. Animal Breeding and Genetics, Agr. College of Norway, Vollebekk. J. Dairy Research, **24**, 316. 1957.

Data from 57 Norwegian Red Poll and 111 Norwegian Red and White Cattle heifers were used to study the relationships between certain milking characteristics. The characteristics studied were machine time, peak flow, per cent yield in 3 min., standard lactation yield, maximum daily yield, and persistency. When the effect of milk yield at the recorded milking was removed, or when only the observations in a narrow range of yield (6.1 to 7.0 kg.) were used, there was no significant effect of milking characters upon lactation yield. It was concluded that slow-milking cows are not necessarily poor milk producers.

J. D. Donker

40. Milking machines. S. Miller. U. S. Patent 2,812,742. 6 claims. Nov. 12, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 2:291. 1957.

Details are given for a milker and its method of attachment to a stall.

R. Whitaker

41. Milking machine with intermittent milk flow. L. Bramley. U. S. Patent 2,814,270. 6 claims. Nov. 26, 1957. Offic. Gaz. U. S. Pat. Office, **724**, 4: 700. 1957.

Details are given for a pipeline milker arrangement employing an air line and a milk line and a milking claw for each milking station. R. Whitaker

42. Milk-line nipple protector. W. T. Pickavance and A. Lea (assignors to National Cooperatives, Inc.). U. S. Patent 2,815,039. 4 claims. Dec. 3, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 1: 67. 1957.

Arrangement is described for capping the outlets of milker pipelines when not in use.

R. Whitaker

43. Pulsated milk-line milking adapter. D. E. Golay (assignor to Farmer Feeder Co.). U. S. Patent 2,809,607. 1 claim. Oct. 15, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 3: 496. 1957.

Structural details are given for the construction of a milker, for attachment to a pipe providing a pulsating vacuum, and to a milk-receiving vessel below the cow.

R. Whitaker

44. Cow stanchion. C. W. Collier. U. S. Patent 2,815,735. 6 claims. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2: 255. 1957.

A cow stanchion which is hinged at the ground level is described. The yoke holding the cow swings forward and backward with the cow's head as the animal stands erect or eats from the trough in front of the stanchion.

R. Whitaker

ICE CREAM

45. Ice cream scoop. R. L. Baer. U. S. Patent 2,815,724. 9 claims. Dec. 10, 1957. Offic. Gaz. U. S. Pat. Office, **725**, 2: 252. 1957.

A hand-operated scoop for forming semi-spherical-shaped portions of ice cream is described. R. Whitaker

46. Boat trailers. L. J. Geil. U. S. Patent 2,809,496. 6 claims. Oct. 15, 1957. Offic. Gaz. U. S. Pat. Office, **723**, 3: 466. 1957.

A small hand-operated ice cream freezer is described. R. Whitaker

NUTRITIVE VALUE OF DAIRY PRODUCTS

47. Lysine in milk proteins in relation to infant needs—A review. F. E. Rice, Evaporated Milk Association, Chicago. J. Am. Dietet. Assoc., **33**, 11: 1141. 1957.

Albanese and coworkers have suggested that it might be desirable to fortify cow's-milk infant-feeding formulas with lysine. This review cites 22 references which demonstrate that the amount of lysine provided by the average evaporated-milk cow's-milk formula is considerably greater than the minimum requirements of the infant as reported from two laboratories. Also, the average cow's-milk formula supplies more lysine than is ingested by the breast-fed baby.

Infant requirement for lysine per kilogram of body weight per day is in the range of 90 to 210 mg. Human milk supplies not more than 165 mg/kg/day, based on the assumption that the breast-fed baby receives 2 to 2.5 g. protein/kg/day. The usual cow's-milk formula prepared from evaporated milk supplies the baby with an estimated 3.5 g. protein/kg/day

which contains from 242 to 273 mg. of lysine.

A protein containing as much as 5.5% lysine is considered to be satisfactorily balanced with respect to this amino acid. Human-milk protein contains an estimated 6.6%, cow's-milk protein 7 to 8% of this amino acid.

The effect of heat on the availability of lysine in cow's-milk protein is reviewed, as is the possible creation of an amino acid imbalance from the proposed lysine fortification.

Frank E. Rice

48. Prophylaxis of allergic disease in infants and children. D. E. JOHNSTONE, Dept. Pediatrics, Univ. of Rochester School of Med., Conn. State Med. J., **21**, 5: 404. 1957.

It has been demonstrated that the pregnant mother can sensitize the fetus through the passage of proteins through the placental barrier. Some of the proteins have their origin in the food the mother ingests. Care of the potentially allergic child rightly begins when it is in utero. A potentially allergic child is defined as one who is either a sibling or an offspring of a person with a major allergy. In some cases the pregnant mother should eat such foods as milk and eggs in an allergenically denatured form, which means the ingestion only of well-boiled milk and eggs. The author recommends also that potentially allergic children get as few solid foods as feasible in the first 4 or 5 mo.

Frank E. Rice

PHYSIOLOGY AND ENDOCRINOLOGY

49. Factors influencing the vitamin content of milk fat. VI. The effect of reduced carotene intake. W. A. MCGILLIVRAY AND N. A. WORKER, Massey Agr. Coll., Palmerston North, New Zealand. J. Dairy Research, **24**, 346. 1957.

Four sets of identical twins in Experiment I and two sets in Experiment II were used to study effect of variation in dietary intake of carotene upon vitamin content of milk fat. Twins in the first experiment were restricted in intake of carotene from a normal of 5 g. daily, according to how much pasture was restricted in different feeding periods. Intake of carotene averaged 2.5, 1.3, and 0.1 g. daily.

In the second experiment, the animals were on vitamin-poor feeds and were given carotene as an oil solution (arachis oil). It was shown that a 50-fold decrease in carotene intake for short periods had only small effects on vitamin A potency of milk. Carotene in arachis oil (750 mg. in 150 ml.) was shown to be relatively more effective than summer pasture, in holding up vitamin A potency of the milk fat.

J. D. Donker

50. Factors influencing the vitamin content of milk fat. VII. The possible existence of protein-bound carotenoids and vitamin A alcohol in mammary secretion. W. A. MCGILLIVRAY, Massey Agr. Coll., Palmerston North, New Zealand. J. Dairy Research, **24**, 352. 1957.

Fats from milk samples were collected either by solvent extraction or by oiling off after preliminary fractionation into fat globules of different sizes by centrifugation. Milk samples were collected from normal cows, cows given extra vitamin A, mastitic cows, and prepartum (colostrum).

The carotenoid content of smaller fat globules was higher than that from larger globules when solvent-extracted. Vitamin A alcohol content of extracted fat varied in a manner similar to carotenoid content, but the reverse appeared to hold for vitamin A ester. The authors suggest that differences in concentration of carotenoids and vitamin A alcohol are not owing to a surface layer on the globule, but to the presence of a protein-bound form of these substances. Mastitic milk had a greater carotenoid and vitamin A alcohol content, and it is suggested that this is owing to these protein-bound forms. Some vitamin A remained in the virtually fat-free serum obtained from normal milk, colostrum, and prepartum secretions, and it was associated with the various serum proteins, particularly the globulin fraction.

J. D. Donker

51. Some properties of the mammary myoepithelium of the sow. W. G. WHITTLESTONE, Ruakura Animal Research Sta., Dept. of Agr., New Zealand. J. Dairy Research, **24**, 321. 1957.

A pectoral and an inguinal mammary gland of sows were used to record simultaneously the effects of administered oxytocin. Pressure recordings were made and the response of the sow's mammary gland was compared to the responses seen from the human uterus when oxytocin was administered. Pressure records of both uterine muscle and mammary myoepithelium are similar, though the pressure developed by the uterus was considerably higher, and the pressure peaks did not fall with time and were further apart in time sequence. A recording apparatus is described.

J. D. Donker

52. Cyclic function in ovarian grafts. R. DEANSLEY, N.I.M.R. Mill Hill, London, N.W.7. J. Endocrinol., **13**, 211. 1956.

Histological examination showed that ovulation occurred normally in autografts and in approximately half of the homografts of rat ovaries. Homografts which were from 7-10 days old donors or which were frozen to −79° C. for 1 hr. had as high a proportion of ovulations as did unfrozen homografts from adult donors.

R. L. Hays

53. Participation of seminal plasma during the passage of spermatozoa in the female reproductive tract of the pig and horse. T. MANN, C. POLGE, AND L. E. A. ROWSON. J. Endocrinol., **13**, 133. 1956.

The ratios of spermatozoa to each of three

characteristic compounds of semen fructose, ergothionine, and citric acid were determined in semen and in the fluids of the uteri and oviducts of sows at 40 min., and a mare at 50 min. after mating. It was found that the sperm and seminal plasma arrive at the uterotubal junction at the same time. In the oviducts of the sow no sperm, fructose, ergothionine, or citric acid was found, but small amounts of these compounds were found in the oviducts of the mare. At 6 hr. after mating, the bulk of sperm and plasma had disappeared from the uteri of sows. R. L. Hays

54. Survival time of ovarian homografts in two strains of rats. A. S. PARKES, N.I.M.R. Mill Hill, London, N.W. 7. J. Endocrinol., **13**, 201. 1956.

Autografts and intra- and inter-strain homografts of ovarian tissue were made subcutaneously in young adult female rats. Vaginal cornification was used to determine the functioning of the grafted tissue. All of the autografts and intra-strain homografts "took," as compared to 16 out of 20 of the inter-strain homografts. After one year, all of the autografts were functional and 11 out of 18 intra-strain and seven of 18 inter-strain homografts were functional. Most grafts contained follicular or lutein cysts, but some normal eggs, follicles, and corpora luteea were found in each type. R. L. Hays

55. Modification of the estrous cycle of the mouse by external stimuli associated with the male. W. K. WHITTEN, John Curtin School of Med. Research, Australian Natl. Univ., Camberra. J. Endocrinol., **13**, 399. 1956.

Of 317 matings of mice during the first five nights after pairing, 45% occurred on the third night. This was approximately three times greater than any other night. The effect was independent of age, parity, or time of day when pairings were made. Mating occurred more frequently on the first night than on any other night if the male was enclosed in a wire basket within the female's cage for two days before pairing. R. L. Hays

56. The response of rabbit mammary glands to locally administered prolactin. T. R. BRADLEY AND P. M. CLARK, N.I.R.D., Univ. Reading, Shinfield, England. J. Endocrinol., **14**, 28. 1956.

The response of rabbit mammary glands to intraductally injected prolactin was investigated with the view of developing an assay for prolactin. Prolactin caused milk secretion by the third day after injection in that sector of the gland into which it was injected in pregnant, pseudopregnant, and estrogen-progesterone treated rabbits. There was some evidence of an increase in response with increased doses, but the variability was high. Pituitary extracts containing the other pituitary hormones did not cause milk secretion. The responses were measured by the presence of milk, weight per unit area, and reducing sugars.

R. L. Hays

57. The effect of scrotal insulation and the influence of the breeding season upon fructose concentration in the semen of the ram. T. D. GLOVER, Unit of Reproductive Physiol. and Biochem., Univ. of Cambridge. J. Endocrinol., **13**, 235. 1956.

Insulation of the scrotum of three rams caused an increase in fructose content of semen from 476 mg. % to 1,000 mg. % in 4 wk. with no effect on libido. After the insulation was removed, the fructose concentration returned to normal in 4–5 wk. At the end of the breeding season, a decrease in fructose concentration occurs immediately before the decrease in the density of sperm and the decline in libido.

R. L. Hays

58. A study of fertilization in the rabbit. The effect of postcoital ligation of the Fallopian tube or uterine horn. C. E. ADAMS, Unit of Reproductive Physiol. and Biochem., Univ. of Cambridge. J. Endocrinol., **13**, 296. 1956.

When ligation was made at the uterotubal junction in 140 females at varying intervals after mating, the percentages of fertilized eggs were 1.6 (1¼ hr.), 8.8 (1¾ hr.), 12.9 (2 hr.), 61.1 (2¼ hr.), 29.8 (2½ hr.), 59.5 (2¾ hr.), 85.9 (3 hr.), 38.4 (3¼ hr.) 73.7 (3½ hr.), 50 (4 hr.), 96.4 (5 hr.), and 100 (6 hr.). In the nonligated horns, 95.5% of the eggs were fertilized. R. L. Hays

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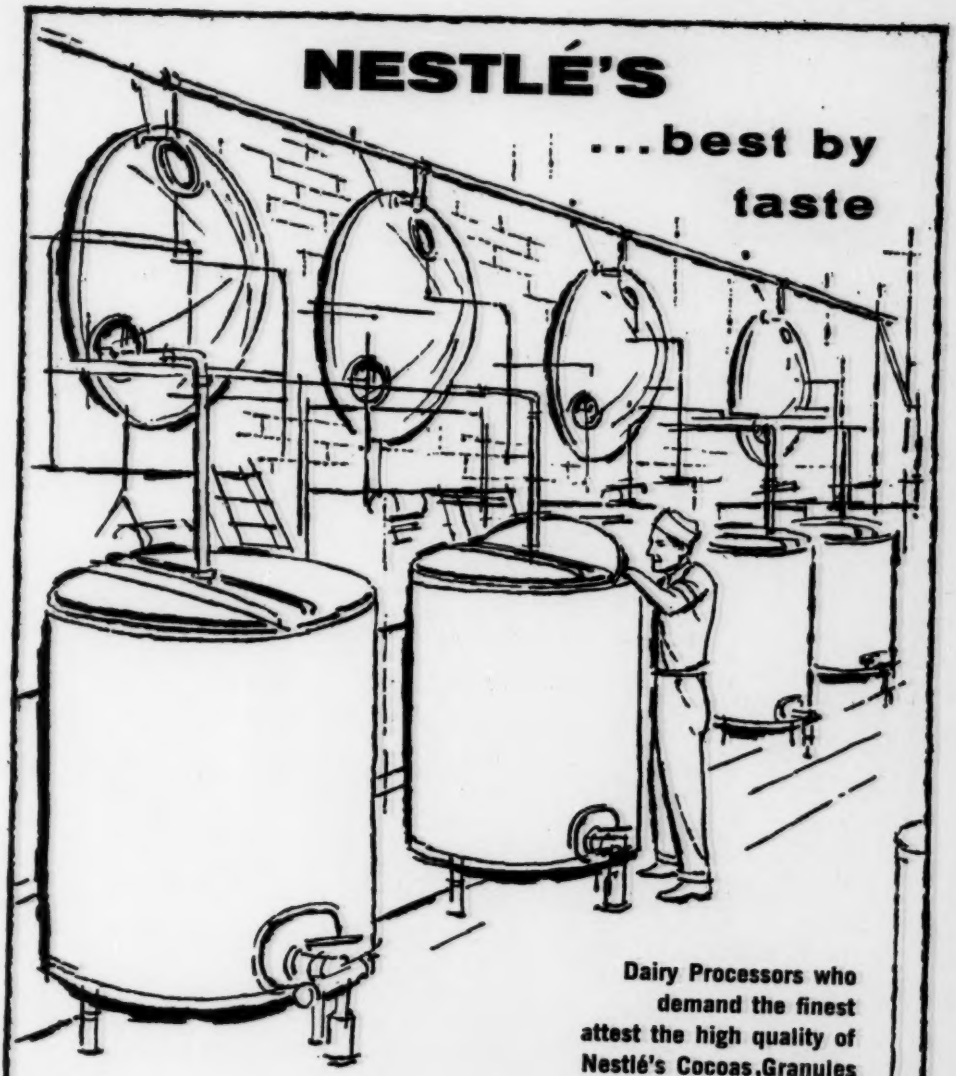

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